

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Application of: Jan Wadstein *et al.*

Serial No.: 09/410,484

Group No.: 1616

Filed: 09/30/99

Examiner: Arnold, Ernst

Entitled: **Method Of Treating Hypertension And Reducing Serum Lipase  
Activity**

**APPELLANTS' BRIEF**

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Appeal Brief - Patents

Commissioner for Patents

P.O. Box 1450

Alexandria, VA 22313-1450

Dear Examiner Ernst:

This Brief is in furtherance of the Notice of Appeal filed May 27, 2008.

Appellants hereby authorize the Commissioner of the Patent and Trademark Office to charge the fees required under § 41.20(b)(2), any required fee for any Petition for Extension of Time, and any other fee for filing this Brief to Attorney Deposit Account No. 50-4302. Please reference Attorney Docket No.: NATNUT-03972 when charging the Attorney Deposit Account.

This Brief contains these items under the following headings and in the order set forth below [37 CFR § 1.192(c)]:

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**I. REAL PARTY IN INTEREST**

The real party in interest is the Assignee, AkerBiomarine ASA, Oslo, Norway.

**II. RELATED APPEALS AND INTERFERENCES**

There are no appeals or interferences related to the pending appeal.

**III. STATUS OF CLAIMS**

Claims 1-18 were filed in the original application. The Claims were subject to a Restriction Requirement mailed December 8, 2000. Applicants elected the Group 1 claims, Claims 1-9, with traverse in a Response dated January 5, 2001. Claims 4, 5, 6, 8 and 10-18 were withdrawn in the Office Action mailed February 20, 2001. Claims 4, 5, and 6 were cancelled in the Response dated November 8, 2004.

Claims 1, 2, 3, 7 and 9 are pending and are being appealed.

Appellants appeal the Final Office Action of January 25, 2008.

The Claims, as they now stand, are set forth in Section VIII. CLAIMS APPENDIX.

**IV. STATUS OF AMENDMENTS**

All previous amendments have been entered.

**V. SUMMARY OF CLAIMED SUBJECT MATTER**

Independent claim 1 is drawn to a method of treating hypertension in humans comprising: a) providing a hypertensive human patient in need of hypertension treatment and a composition comprising a safe and effective amount conjugated linoleic acid for treating hypertension; and b) administering said conjugated linoleic acid composition to said human patient so that blood pressure of said human patient is reduced.

This method is described in the specification on page 3, lines 23-27, which provides that that in some embodiments of the invention, a conjugated linoleic acid composition is given to a subject under conditions such that the blood pressure of the subject is reduced. The method is further described in the specification at page 10, lines 5-7, which provides that "the data described below in Example 1 indicates that dietary supplementation with CLA results in a decrease in both systolic and diastolic blood pressure in humans with borderline mild hypertension." Page 11, lines 8-15, further provides that:

In a typical regimen, an individual will begin the hypertension treatment program or serum lipase activity reduction program by ingesting up to several grams (e.g., 0.1 to 5

grams) of CLA with each meal, and monitoring blood pressure over a period of several months. This CLA may be provided in the form of a pill or as a component of a prepared food product. Once the desired blood pressure or serum lipase activity has been attained, a proper maintenance level can be found by gradually reducing the dose and continuing to monitor blood pressure to assure there is no increase.

Dependent Claim 2 specifies the method of Claim 1 wherein the conjugated linoleic acid composition is a mixture of octadecadienoic acid isomers selected from the group of cis-9, trans-11; cis-9, cis-11; trans-9, cis-11; trans-9, trans-11; cis-10, cis-12; cis-10, trans-12; trans-10, cis-12; trans-10, trans-12 octadecadienoic acid. Octadecadienoic acid is another name for conjugated linoleic acid or CLA. The isomers of conjugated linoleic acid are discussed in detail in the specification at page 8, line 25, to page 9, line 28.

Dependent Claim 3 specifies the method of Claim 1 wherein the conjugated linoleic acid composition consists essentially of octadecadienoic acid isomers selected from 9,11 octadecadienoic acid, 10,12 octadecadienoic acid, and mixtures thereof. These preferred isomers of CLA are discussed in detail in the specification at page 9, lines 13-28.

Dependent Claim 7 specifies the method of Claim 1 wherein the conjugated linoleic acid is administered orally. Administration of CLA is discussed in detail in the specification at page 10, line 28 to page 11, line 15 of the specification.

Dependent Claim 9 of the specification specifies the method of Claim 1 wherein said safe and effective amount of conjugated linoleic acid is about 0.1 grams to 20 grams. Dosages of CLA are discussed in detail in the specification at page 11, lines 8-21.



**VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL**

There is one grounds of rejection to be reviewed on appeal:

Whether Claims 1-3, 7 and 9 are obvious over Cook et al. (U.S. Pat. No. 5,554,646) in view of Kawamura et al. (Hypertension 1996, 27, 408-413) and Shinitzky (U.S. Pat. No. 4,474,773).

## **VII. ARGUMENT**

### **A. The Claims are Not Obvious**

Claims 1-3, 7 and 9 stand rejected as obvious over Cook et al. (U.S. Pat. No. 5,554,646) in view of Kawamura et al. (Hypertension 1996, 27, 408-413) and Shinitzky (U.S. Pat. No. 4,474,773). The Office has found that:

- Cook et al. disclose a method of reducing body fat comprising the administration of a safe and effective amount of conjugated linoleic acid (Final Office Action mailed Jan. 25, 2008 at 3);
- Kawamura et al. provide a nexus teaching between hypertension, weight loss and decreases in blood pressure and that changes in body weight exhibited significant correlations with blood pressure in hypertensive overweight human patients (Id. at 3);
- Shinitzky et al. teach treating hypertension by administering a composition comprising 5-10% linoleic acid (Id. at 3).

The Office admits that the primary reference, Cook et al., does not teach a method of administering CLA to treat hypertension in humans. Id. at 4. The Office goes on to argue, however, that one of ordinary skill would have been motivated to combine the references to arrive at the claimed invention because:

- Cook et al. teaches a method of reducing body fat;
- Kawamura et al. teach that reduction of weight of hypertensive patients results in a lowering of blood pressure;
- Shinitzky et al. teach that linoleic acid (C18:2, cis-9, cis-12) can be used to treat hypertension; and
- Since conjugated linoleic acid is a mixture of positional and geometric isomers of linoleic acid, then one of skill in the art would immediately envision conjugated linoleic acid in the treatment of hypertension.

In rejecting claims under 35 U.S.C. § 103, the Examiner bears the initial burden of presenting a *prima facie* case of obviousness. A *prima facie* case of obviousness requires the Examiner to cite a combination of references which (a) disclose the elements of the claimed invention, (b) suggests or motivates one of skill in the art to combine those elements to yield the claimed combination, and (c) provides a reasonable expectation of success should the claimed combination be carried out. Failure to establish any one of these three requirements precludes a finding of a *prima facie* case of obviousness, and, without more, entitles Applicant to

allowance of the claims at issue. *See, e.g., Northern Telecom Inc. v. Datapoint Corp.*, 15 USPQ2d 1321, 1323 (Fed. Cir. 1990).

When a *prima facie* case is made (for the record, Applicants do not believe a *prima facie* case has been made here), the burden shifts to the applicant to come forward with evidence and/or argument supporting patentability. *In re Sullivan*, 498 F. 3d 1345, 1351, 81 USPQ2d 1034 (Fed. Cir. 2007) (citing *In re Glaug*, 283 F.3d 1335, 1338 (Fed.Cir.2002)). As held in *Sullivan*:

Rebuttal evidence is “merely a showing of facts supporting the opposite conclusion.” *In re Piasecki*, 745 F.2d 1468, 1472 (Fed.Cir.1984). Evidence rebutting a *prima facie* case of obviousness can include: “evidence of unexpected results,” *Pfizer, Inc. v. Apotex, Inc.*, 480 F.3d 1348, 1369 (Fed.Cir.2007), evidence “that the prior art teaches away from the claimed invention in any material respect,” *In re Peterson*, 315 F.3d 1325, 1331 (Fed.Cir.2003), and evidence of secondary considerations, such as commercial success and long-felt but unresolved needs, *WMS Gaming, Inc. v. Int’l Game Tech.*, 184 F.3d 1339, 1359 (Fed.Cir.1999).

*Id.* Importantly, when a patent applicant puts forth rebuttal evidence, the Office must consider that evidence. *Id., see also In re Soni*, 54 F.3d 746, 750 (Fed.Cir.1995) (stating that “all evidence of nonobviousness must be considered when assessing patentability”); *In re Sernaker*, 702 F.2d 989, 996 (Fed.Cir.1983) (“If, however, a patent applicant presents evidence relating to these secondary considerations, the board must always consider such evidence in connection with the determination of obviousness.”).

The determination whether an invention would have been obvious under 35 U.S.C. § 103 is a legal conclusion based on underlying findings of fact. *Id.* at 1350 (citing *In re Kotzab*, 217 F.3d 1365, 1369 (Fed.Cir.2000)). With respect to the instant invention, the Office has failed to establish a *prima facie* case evidence because the facts the Office has relied on are scientifically incorrect or inaccurate and, in any event, has failed to considered properly established facts submitted in rebuttal. In particular, the Office has made scientifically incorrect and unsupportable assumptions about the teachings of Cook and Shinitzky and has failed to consider pertinent rebuttal evidence established in the Declarations of Mr. Asgeir Saebo and Dr. Inge Bruheim. Applicants detail these errors below and request that the obviousness rejection be withdrawn because of these errors.

**1. Shinitzky does not teach that linoleic acid can be used to treat hypertension**

The Office alleges that Shinitzky et al. teaches that linoleic acid (C18:2, cis-9, cis-12) can be used to treat hypertension. This greatly overstates the teachings of Shinitzky et al. to one of skill in the art. In making this finding, the Examiner relies on the claims of Shinitzky et al. Examination of the claims reveals, however, that Shinitzky et al. utilizes a complex lipid mixture that contains linoleic acid and provides no indication that linoleic acid is the active ingredient. This issue is specifically addressed in the Bruheim Declaration, provided in Section IX below at p. 20-76.

The Office specifically refers to Claims 1, 4, and 24 of Shinitzky as providing the teaching that linoleic acid can be used to treat hypertension. Office Action mailed June 26, 2007 at 3; Office Action mailed Jan. 25, 2008 at 6. What those claims teach is that hypertension can be treated by administering a complex mixture comprising "a lipid fraction derived from natural sources (AL), said lipid fraction containing 40-80 weight percent glycerides, 3-5 weight percent cholesterol, 10-30 weight percent lecithin (phosphatidyl choline), 5-15 weight percent phosphatidyl ethanolamine and 2-5 weight percent negatively charged phospholipids, wherein the ratio of unsaturated to saturated fatty acids is at least 1:1" (Claim 1) and that the fatty acid component of this complex mixture comprises "Palmitic acid 35-45%, oleic acid 35-45%, linoleic acid 5-10%, stearic acid 5-7%, palmitoleic acid 2-3%, arachidonic acid 0.2-1%" (Claim 4). *See* Shinitzky et al., Claims 1 and 4 and Bruheim Decl. at ¶2. As can be plainly seen, the invention claimed in claims 1, 4, and 24 utilizes a complex lipid mixture, of which linoleic acid is only a small part. Thus, it is scientifically incorrect and inaccurate to state that Shinitzky et al. teaches that linoleic acid can be used to treat hypertension. This statement by the Office is not factually supported.

The Bruheim Declaration directly addresses this issue. As established by Dr. Bruheim:

- Shinitzky et al. does not teach that linoleic acid can be used to treat hypertension or that among all of the components of the complex mixture, linoleic acid is sufficient to treat hypertension.
- Shinitzky et al. teaches that a complex lipid fraction can be used to treat hypertension.
- This is not the same as teaching that linoleic acid can be used to treat hypertension.
- One of skill in the art would recognize that any of the other components could be responsible for the hypertensive effect or that a combination of the components is necessary.

Bruheim Decl. ¶2 (p. 20 in Section IX below). The Office provides no factual or scientifically based rebuttal of these facts in the Office Action mailed January 25, 2008. The Office argues that “it is the Examiner’s position that each component is treating hypertension because that is what the claim language clearly states. There can be no other interpretation of the claim.” *Id.* at 6. This is simply incorrect. Neither the claims nor the specification of Shinitzky teach that each component of the complex lipid mixture treats hypertension.

The Office goes on to argue that “Shinitzky et al. do not have to recite a method that utilizes solely linoleic acid. The Examiner also notes for the record the open claim language of the instant application.” *Id.* at 7. Apparently, the Office is trying to make an argument based on claim interpretation. Appellants note however, the current claims are limited to the use of conjugated linoleic acid, which is a mixture of octadecadienoic acid isomers such as cis-9, trans-11; cis-9, cis-11; trans-9, cis-11; trans-9, trans-11; cis-10, cis-12; cis-10, trans-12; trans-10, cis-12; trans-10, trans-12 octadecadienoic acid. The claims do not encompass linoleic acid, which, as noted by the Examiner, is c9, c12 octadecadienoic acid. Linoleic acid does not have a conjugated double bond. Since the use of a different chemical entity is being claimed, i.e., the use of conjugated linoleic acid as opposed to non-conjugated linoleic acid, then the scientific issue of whether Shinitzky teaches that each component of the complex lipid preparation can be used to treat hypertension is relevant and is not addressed by interpreting the claims broadly. If a person of skill in the art set out to make a composition to treat hypertension based the information in the Shinitzky claims, that person would have no guidance to use linoleic acid, much less conjugated linoleic acid. This is confirmed by the Bruheim Declaration.

Furthermore, Appellants note another key flaw in the Office’s analysis. Cook et al. teaches that administration of conjugated linoleic acid, and **not** linoleic acid, reduces body fat. Cook et al., column 2, lines 48-67. The Office’s reasoning is that one would be motivated to use CLA to treat hypertension because reductions in body weight cause hypertension to be reduced. If, as taught by Cook et al., linoleic acid does not reduce body weight, then why would a person of skill in the art, having knowledge of Cook, think that the linoleic acid in the complex Shinitzky lipid preparation, could be used to treat hypertension? Thus, Cook et al. **teaches away** from combination with Shinitzky et al. Accordingly, it is improper to combine Cook et al. with Shinitzky in the first place.

**2. The Office has not rebutted the fact established by Appellants that linoleic acid and conjugated linoleic acid have different properties**

The Office's second error is its failure to consider the evidence presented in the Bruheim Declaration that establishes that the different isomers of linoleic acid have different properties. In the Office Action mailed June 26, 2007 at page 5, the Office argues that "it remains obvious to one of ordinary skill in the art that the method of Cook et al. can lower blood pressure via administration of conjugated linoleic acid because the positional and geometric isomers of linoleic acid would be expected to have similar properties to linoleic acid in the absence of evidence to the contrary." This argument is related to the Office's *In re Dillon* argument on pages 3 and 4 of the Office Action that chemical compounds with similar structures would be expected by those of skill in the art to similar utilities.

However, the Bruheim Declaration directly rebuts the assumption that linoleic acid and conjugated linoleic acid have similar properties. In particular, Dr. Bruheim establishes that:

- Conjugated isomers of conjugated linoleic acid have different biological properties than linoleic acid.
- This was known in the art prior to the filing date of this application and has been substantiated in many publications after the filing date of this application.
- The references attached to the Bruheim Declaration establish that the conjugated isomers of linoleic acid have distinct biological properties as compared to standard linoleic c9,c12 linoleic acid.
- Many of these references utilize corn oil, which comprises c9,c12 linoleic acid, as a control.
- All of the references show that the conjugated linoleic acid isomers have distinct biological properties.
- Dr. Mark Cook, the inventor of the cited 5,554,646 patent, is an author on most of these publications.

Bruheim Declaration ¶13 (p. 21 in Section IX below). Thus, Appellants provided evidence that establishes that conjugated linoleic acid and linoleic acid have different properties.

In the Office Action mailed January 25, 2008 at page 7, the Office argued that these facts are not persuasive because Cook et al. teach that free linoleic acid is converted to conjugated linoleic acid in the animal. (Citing Column 3, lines 52-56 of Cook et al.). That portion of Cook et al. provides that "The linoleic acid is converted to CLA in the animal, probably by microorganisms in the animal's gastrointestinal system (S.F. Chin, J. M. Storkson, W. Liu, K. Albright, and M. W. Pariza, 1994, J. Nutr. 124:694-701). This assertion by the Office is irrelevant and scientifically incorrect as applied to humans. The Chin et al. reference is attached to the Bruheim Declaration. Chin et al. plainly teaches that linoleic acid is converted to

conjugated linoleic acid by rumen microorganisms at p. 2344 Column 2 (p. 65 in Section IX below), which provides that:

Kepler et al. [1966] identified the cis-9, trans-11 CLA isomer as an intermediate in the biohydrogenation of linoleic acid by the rumen bacterium *Butyrivibrio fibrisolvens*. Seemingly, some of the CLA produced in this way escapes conversion to stearic acid, is absorbed from the digestive system, and is subsequently incorporated into tissue lipid (including milk phospholipid).

There is no teaching in Cook et al. or Chin et al. that humans can convert linoleic acid into conjugated linoleic acid which is what the Office is attempting to establish. The conversion occurs in rumen microorganisms which are not present in the human gut. Accordingly, the Office has failed to rebut the evidence of the Appellants that establishes that conjugated linoleic acid has different effects than linoleic acid. The Office argues that “any linoleic acid administered to an animal would be expected to be converted into conjugated linoleic acid and have all of the benefits of action of conjugated linoleic acid as per the teachings of Cook et al.” Office Action mailed January 25, 2008 at 7. This argument is clearly incorrect with respect to humans, which the claims are limited to. Of course, this very point is also established by Cook et al. which teaches that conjugated linoleic acid causes a reduction in body fat while linoleic acid does not. If humans were able to convert linoleic acid into conjugated linoleic acid there would be no need for the claimed invention of Cook which is administration of conjugated linoleic acid to a human for the purpose of reducing body fat.

This scientifically irrelevant argument is the *only* evidence offered by the Office to rebut ¶ 3 of the Bruheim Declaration and the facts contained therein, which are summarized above. Thus, the fact that a person of ordinary skill in the art would recognize that linoleic acid and conjugated linoleic acid have different properties as demonstrated by multiple scientific papers is unrebutted. As a result, the Office’s *In re Dillon* argument is inapplicable and properly rebutted.

**3. The Office failed to rebut the facts established in the Saebo Declaration that agents that cause weight loss can cause hypertension and that CLA could be expected to cause hypertension**

The third error made by the Office is the failure to consider the rebuttal evidence in the Saebo Declaration, provided in full in Section IX below (p. 77-105), that establishes that 1) it is incorrect to make an assumption that a compound that reduces weight will also cause a reduction in hypertension and 2) the art indicates that CLA would be expected to have a hypertensive effect.

The Saebo Declaration establishes that:

- When a biologically active agent, such as CLA, is administered to a subject there can be a variety of effects.
- Just because CLA causes weight loss does not also mean that it would reduce hypertension.
- A person of skill in the art would not reasonable expect CLA to reduce hypertension for two reasons.
- CLA has been shown to elevate the level of F2-isoprostane. Taylor et al., Conjugated Linoleic Acid Impairs Endothelial Function, Arteriosclerosis, Thrombosis, and Vascular Biology 26(2), 307-312 (2006)(attached to Declaration).
- F2-isoprostanes have a vasoconstrictive effect. Cracowski et al., Cardiovascular pharmacology and physiology of the isoprostanes, Fundamental & Clinical Pharmacology 20(5): 417-427 (2006)(attached to Declaration).
- Taken together, it should be expected that administration of CLA would result in an increase in blood pressure.
- Second, administration of other agents known to be effective for weight loss can result in increased hypertension.
- Ephedrine, a commonly used, biologically active weight loss supplement is one such example.
- As established in Haller and Benowitz, Adverse Cardiovascular and Central Nervous System Events Associated with Dietary Supplements Containing Ephedra Alkaloids, New England J. Med. 343(25):1833-1838 (2000)(attached to Declaration), ephedrine can cause an increase in hypertension.
- The Examiner's argument that it would be obvious to use CLA to decrease hypertension because CLA administration also causes weight loss lacks scientific merit.
- How an agent such as CLA acts in the body is complex.
- Whether CLA causes an increase or decrease in hypertension, or has no effect at all, is determined by a variety of factors that have no relation to weight loss.
- It is not scientifically valid to draw a conclusion that because an agent causes weight loss, it can also be expected to decrease hypertension.
- The references cited by the Examiner contain no data that can be interpreted in this manner.

Saebo Declaration ¶3-6 (p. 78 in Section IX below). The Examiner addressed the Saebo Declaration in the Office Action dated June 26, 2007, at page 5. First, the Office **completely failed** to rebut ¶4 of the Saebo Declaration which establishes that CLA would be expected to



increase, not decrease hypertension because of its effect of elevating levels F2-isoprostane. Second, even though the Office recognized other parts of the Saebo Declaration, the Office's arguments are not relevant and do nothing to rebut the facts established in the Saebo Declaration. In attempting to rebut the Saebo Declaration, the Office argues that:

First, Kawamura et al. provide a nexus teaching between weight loss in hypertensive patients and lowering of blood pressure. Cook et al. provide a method of reducing body fat, which is weight loss. Shinitzky et al. teach linoleic acid in the treatment of hypertension. The art recognizes linoleic acid in a method of treating hypertension. (See also pertinent art below.) Therefore, it remains obvious to one of ordinary skill in the art that the method of Cook et al. can lower blood pressure via administration of conjugated linoleic acid because the positional and geometric isomers of linoleic acid would be expected to have similar properties to linoleic acid in the absence of evidence to the contrary.

Office Action dated June 26, 2007, at page 5 (emphasis by the Office). This "rebuttal" merely restates the arguments already made by the Office in attempting to establish a *prima facie* case of obviousness. There is no rebuttal of the actual facts in the Saebo Declaration. For example, there is no rebuttal of the fact that biologically active agents that cause weight loss can also cause hypertension. This is an important point. When a biologically active agent is used to induce weight loss, the agent can have other effects unrelated to the weight loss. There is a difference between weight loss caused by a biologically active agent and weight loss due to dietary changes or exercise. Likewise, there is no rebuttal of the fact that CLA has been shown to elevate F2-isoprostane which can cause hypertension. Applicants further note that, as discussed above, the Bruheim Declaration established that linoleic acid and conjugated linoleic acid cannot be expected to have similar properties as alleged by the Office.

The Office's primary reasoning supporting its alleged *prima facie* case of obviousness is that Kawamura et al. provides a nexus teaching between hypertension, weight loss and decreases in blood pressure. Office Action mailed Jan. 25, 2008 at 3. Thus, accordingly to the Office's reasoning, any agent that reduces body weight would also decrease hypertension. The Saebo Declaration directly rebuts the Office's incorrect reasoning.

**4. Taken together, these facts rebut any *prima facie* case of obviousness established by the Office**

As demonstrated above, to the extent that any *prima facie* case of obviousness was established by the Office, it was rebutted by the factual evidence presented during prosecution. The Office's alleged *prima facie* of obviousness is based on the assertions that:

- Cook et al. teaches a method of reducing body fat;
- Kawamura et al. teach that reduction of weight of hypertensive patients results in a lowering of blood pressure;
- Shinitzky et al. teach that linoleic acid (C18:2, cis-9, cis-12) can be used to treat hypertension; and
- Since conjugated linoleic acid is a mixture of positional and geometric isomers of linoleic acid, then one of skill in the art would immediately envision conjugated linoleic acid in the treatment of hypertension.

First, Applicants have demonstrated above that this is not a proper *prima facie* of obviousness, because Cook et al. actually teaches away from combination with Shinitzky because Cook et al. teaches that CLA is effective to reduce body fat, not linoleic acid. Thus, a person of skill in the art would not believe that the linoleic acid in Shinitzky's complex lipid mixture was effective for reducing body fat or, as argued by the Office, hypertension. Moreover, the facts established by Appellants demonstrate that a person of skill in the art would have expected CLA to have a hypertensive effect, not the effect of reducing hypertension. These facts strongly support the non-obviousness of the invention as claimed under the *Graham* factors.

Second, to the extent that a *prima facie* case of obviousness was presented by the Office, Appellants have presented factual evidence that rebuts the Office's arguments in the form of the Saebo and Bruheim Declarations. Many of the facts in these Declarations were not even addressed by the Office and the Office's arguments regarding the remaining facts are scientifically incorrect and insufficient to rebut the facts contained in the Declarations.

Taken together, these Declarations establish the following:

- Shinitzky et al. does not teach that linoleic acid can be used to treat hypertension or that among all of the components of the complex mixture, linoleic acid is sufficient to treat hypertension. Bruheim Declaration ¶ 2 (p. 20 in Section IX below).
- Shinitzky et al. teaches that a complex lipid fraction can be used to treat hypertension. *Id.*
- This is not the same as teaching that linoleic acid can be used to treat hypertension. *Id.*
- One of skill in the art would recognize that any of the other components could be responsible for the hypertensive effect or that a combination of the components is necessary. *Id.*

- Conjugated isomers of conjugated linoleic acid have different biological properties than linoleic acid. Bruheim Declaration ¶ 3 (p. 21 in Section IX below).
- This was known in the art prior to the filing date of this application and has been substantiated in many publications after the filing date of this application. *Id.*
- The references attached to the Bruheim Declaration establish that the conjugated isomers of linoleic acid have distinct biological properties as compared to standard linoleic c9,c12 linoleic acid. *Id.*
- Many of these references utilize corn oil, which comprises c9,c12 linoleic acid, as a control. *Id.*
- All of the references show that the conjugated linoleic acid isomers have distinct biological properties. *Id.*
- Dr. Mark Cook, the inventor of the cited 5,554,646 patent, is an author on most of these publications. *Id.*
- When a biologically active agent, such as CLA, is administered to a subject there can be a variety of effects. Saebo Declaration ¶ 3 (p. 78 in Section IX below).
- Just because CLA causes weight loss does not also mean that it would reduce hypertension. *Id.*
- A person of skill in the art would not reasonable expect CLA to reduce hypertension for two reasons. *Id.*
- CLA has been shown to elevate the level of F2-isoprostane. Taylor et al., Conjugated Linoleic Acid Impairs Endothelial Function, Arteriosclerosis, Thrombosis, and Vascular Biology 26(2), 307-312 (2006). Saebo Declaration ¶ 4 (p. 78 in Section IX below), *see* p. 94-99 of Evidence Appendix for Taylor et al.
- F2-isoprostanes have a vasoconstrictive effect. Cracowski et al., Cardiovascular pharmacology and physiology of the isoprostanes, Fundamental & Clinical Pharmacology 20(5): 417-427 (2006). *Id.*, *see* p. 81-91 of Evidence Appendix for Cracowski et al.
- Taken together, it should be expected that administration of CLA would result in an increase in blood pressure. Saebo Declaration ¶ 4 (p. 78 in Section IX below).
- Second, administration of other agents known to be effective for weight loss can result in increased hypertension. Saebo Declaration ¶ 5 (p. 78 in Section IX below).
- Ephedrine, a commonly used, biologically active weight loss supplement is one such example. *Id.*

- As established in Haller and Benowitz, Adverse Cardiovascular and Central Nervous System Events Associated with Dietary Supplements Containing Ephedra Alkaloids, New England J. Med. 343(25):1833-1838 (2000), ephedrine can cause an increase in hypertension. *Id.*, see p. 100-105 of Evidence Appendix for Haller and Benowitz.
- The Examiner's argument that it would be obvious to use CLA to decrease hypertension because CLA administration also causes weight loss lacks scientific merit. Saebo Declaration ¶ 5.
- How an agent such as CLA acts in the body is complex. *Id.*
- Whether CLA causes an increase or decrease in hypertension, or has no effect at all, is determined by a variety of factors that have no relation to weight loss. *Id.*
- It is not scientifically valid to draw a conclusion that because an agent causes weight loss, it can also be expected to decrease hypertension. *Id.*
- The references cited by the Examiner contain no data that can be interpreted in this manner. *Id.*

These facts are essentially un rebutted by the Office and rebut any *prima facie* case of obviousness established by the Office. Moreover, Appellants have established that there is no teaching in Cook et al. or Chin et al. that humans can convert linoleic acid into conjugated linoleic acid which is what the Office is attempting to establish which is one of the key rebuttal arguments made by the Office. The conversion occurs in rumen microorganisms which are not present in the human gut. The claimed invention is non-obvious and the rejection should be withdrawn.

## **VIII. CLAIMS APPENDIX**

1. (Previously presented) A method of treating hypertension in humans comprising:  
a) providing a hypertensive human patient in need of hypertension treatment and a composition comprising a safe and effective amount conjugated linoleic acid for treating hypertension; and  
b) administering said conjugated linoleic acid composition to said human patient so that blood pressure of said human patient is reduced.

2. (Previously presented) The method of Claim 1 wherein the conjugated linoleic acid composition is a mixture of octadecadienoic acid isomers selected from the group of cis-9, trans-11; cis-9, cis-11; trans-9, cis-11; trans-9, trans-11; cis-10, cis-12; cis-10, trans-12; trans-10, cis-12; trans-10, trans-12 octadecadienoic acid.

3. (Previously presented) The method of Claim 1 wherein the conjugated linoleic acid composition consists essentially of octadecadienoic acid isomers selected from 9,11 octadecadienoic acid, 10,12 octadecadienoic acid, and mixtures thereof.

4-6. Cancelled.

7. (Previously presented) The method of Claim 1 wherein the conjugated linoleic acid is administered orally.

8. (withdrawn) The method of Claim 1, wherein the conjugated linoleic acid is provided in a prepared food product.

9. (Previously presented) The method of Claim 1 wherein said safe and effective amount of conjugated linoleic acid is about 0.1 grams to 20 grams.

10. (Withdrawn) A method of reducing serum lipase activity in humans comprising  
a) providing a subject and a composition comprising a safe and effective amount conjugated linoleic acid; and  
b) administering said conjugated linoleic acid composition to said subject under conditions such that serum lipase activity of said subjects is reduced.

11. (Withdrawn) The method of Claim 10 wherein the conjugated linoleic acid composition is a mixture of octadecadienoic acid isomers selected from the group of cis-9, trans-11; cis-9, cis-11; trans-9, cis-11; trans-9, trans-11; cis-10, cis-12; cis-10, trans-12; trans-10, cis-12; trans-10, trans-12 octadecadienoic acid.

12. (Withdrawn) The method of Claim 10 wherein the conjugated linoleic acid composition consists essentially of octadecadienoic acid isomers selected from 9,11 octadecadienoic acid, 10,12 octadecadienoic acid, and mixtures thereof.

13. (Withdrawn) The method of Claim 10 wherein said conjugated linoleic acid composition comprises esters of conjugated linoleic acid.

14. (Withdrawn) The method of Claim 13 wherein said esters are selected from methyl esters and ethyl esters.

15. (Withdrawn) The method of Claim 10 wherein said conjugated linoleic acid composition comprises triglycerides including at least one conjugated linoleic acid at the SN-1, SN-2, or SN-3 position of said triglycerides.

16. (Withdrawn) The method of Claim 10 wherein the conjugated linoleic acid is administered orally.

17. (Withdrawn) The method of Claim 10 wherein the conjugated linoleic acid is provided in a prepared food product.

18. (Withdrawn) The method of Claim 10 wherein said safe and effective amount of conjugated linoleic acid is about 0.1 to 20 grams.

**IX. EVIDENCE APPENDIX**

This evidence appendix contains the Declarations of Dr. Inge Bruheim and Mr. Asgeir Saebo. The Bruheim Declaration was entered by the Examiner in the Office Action mailed January 25, 2008. The Saebo Declaration was entered by the Examiner in the Office Action mailed September 26, 2007.

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Application of:	Jan Wadstein <i>et al.</i>	
Serial No.:	09/410,484	Group No.: 1614
Filed:	09/30/99	Examiner: Webman
Entitled:	<b>Method Of Treating Hypertension And Reducing Serum Lipase Activity</b>	

**Declaration of Dr. Inge Bruheim**

EFS WEB-FILED

Assistant Commissioner for Patents  
Washington, D.C. 20231

I, Dr. Inge Bruheim, state as follows:

1. My present position is Senior Research Scientist, Aker Biomarine AS, the current owner of the instant patent application. I have a Ph.D. in Chemistry from the University of Oslo (Norway) and more than 4 years of industry experience in chemical research.
  
2. The Examiner states on page 3 of the Office Action that Shinitzky et al. teaches that linoleic acid (C18:2, cis-9,cis-12) can be used to treat hypertension. One of skill in the art would not interpret Shinitzky as providing this teaching. The Examiner specifically refers to Claims 1, 4, and 24 of Shinitzky as providing the teaching that linoleic acid can be used to treat hypertension. What those claims teach is that hypertension can be treated by administering a complex mixture comprising "a lipid fraction derived from natural sources (AL), said lipid fraction containing 40-80 weight percent glycerides, 3-5 weight percent cholesterol, 10-30 weight percent lecithin (phosphatidyl choline), 5-15 weight percent phosphatidyl ethanolamine and 2-5 weight percent negatively charged phospholipids, wherein the ratio of unsaturated to saturated fatty acids is at least 1:1" (Claim 1) and that the fatty acid component of this complex mixture comprises "Palmitic acid 35-45%, oleic acid 35-45%, linoleic acid 5-10%, stearic acid 5-7%, palmitoleic acid 2-3%, arachidonic acid 0.2-1%" (Claim 4). Shinitzky et al. does not teach that linoleic acid can be used to treat hypertension or that among all of the components of the



complex mixture, linoleic acid is sufficient to treat hypertension. Shinitzky et al. teaches that complex lipid fraction can be used to treat hypertension. This is not the same as teaching that linoleic acid can be used to treat hypertension. Indeed, one of skill in the art would recognize that any of the other components could be responsible for the hypertensive effect or that a combination of the components is necessary.

3. The Examiner further states the positional and geometric isomers of linoleic acid would be expected to have similar properties to linoleic acid in the absence of evidence to the contrary. Isomers of conjugated linoleic acid have different biological properties than linoleic acid. This was known in the art prior to the filing date of this application and has been substantiated in many publications after the filing date of this application. I have included several references, which are attached, that establish that the conjugated isomers of linoleic acid have distinct biological properties as compared to standard linoleic c9,c12 linoleic acid. Many of these references utilize corn oil, which comprises c9,c12 linoleic acid, as a control. All of the references show that the conjugated linoleic acid isomers have distinct biological properties. Furthermore, Dr. Mark Cook, the inventor of the cited 5,554,646 patent, is an author on most of these publications.

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**PATENT**

U.S. Appln. Ser. No.: 09/410,484  
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4. I further declare that all statement made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.



Dr. Inge Bruheim

Date: 23/10 - 2007

FEEDING CONJUGATED LINOLEIC ACID TO ANIMALS PARTIALLY  
OVERCOMES CATABOLIC RESPONSES DUE TO ENDOTOXIN INJECTION

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Received December 25, 1993

**Summary:** The ability of conjugated linoleic acid to prevent endotoxin-induced growth suppression was examined. Mice fed a basal diet or diet with 0.5% fish oil lost twice as much body weight after endotoxin injection than mice fed conjugated linoleic acid. By 72 hours post injection, mice fed conjugated linoleic acid had body weights similar to vehicle injected controls; however, body weights of basal and fish oil fed mice injected with endotoxin were reduced. Conjugated linoleic acid prevented anorexia from endotoxin injection. Splenocyte blastogenesis was increased by conjugated linoleic acid.

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Recently, nonessential fatty acids have been shown to modulate acute inflammatory and immune responses possibly by changing cellular fatty acid profiles (1). The ability of two major fatty acid components of fish oil, eicosapentaenoate (EPA) and docosahexaenoate (DHA), to modulate the synthesis of arachidonate and its metabolites was recognized as one mechanism of immune regulation by fatty acids. EPA has been shown to depress arachidonic acid derived prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) synthesis in peritoneal leukocytes from rats (2). Unlike corn oil-fed rats, fish oil-fed rats did not exhibit the characteristic depression in food intake when injected with interleukin-1 (IL-1) (3). This phenomena has been partially attributed to the ability of fish oil to block or alter the cyclooxygenase pathway thereby decreasing PGE<sub>2</sub> synthesis and altering the ability of PGE<sub>2</sub> to negatively feed back on IL-1 synthesis.

CLA (conjugated dienoic derivative of linoleic acid) was shown to be a naturally occurring substance in food which has an anticarcinogenic effect on dimethylbenz(a)anthracene induced

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mammary tumors (4). Recent studies (5) have shown that CLA decreases arachidonic acid content of select tissues. It was postulated that like fish oil, CLA may exert an effect on the immune system by altering product formation of either the cyclooxygenase or lipoxygenase pathway. The purpose of the following investigation was to determine the effect of CLA on endotoxin induced growth inhibition and food intake depression. Fatty acid levels in tissue and *in vitro* lymphocyte proliferation was examined.

#### Materials and methods

Preparation of CLA and purity determination: CLA was prepared from linoleic acid by alkali isomerization as previously described (6, 7). Purity was determined to exceed 97%. CLA was stored in an argon atmosphere at -20°C.

Tissue fatty acid determination: Tissue fatty acid levels were determined by the following procedure. Total fat was extracted by chloroform:methanol (2:1, v/v) as described by Folch et al (8). Fatty acid methyl esters were prepared by reaction with 4% HCL in methanol for 20 minutes at 60 C (9) and extracted with hexane. Tridecanoic acid methyl ester was used as an internal standard and fatty acid methyl esters were identified by comparison with standards using gas chromatography (Hewlett Packard 5890 series II) (7).

Mouse experimental protocol: Three-week old mixed-sex mice (U.W. Madison Dairy Science Dept. outbred colony) (4 per group and 3 groups per dietary treatment) were fed a semi-purified basal diet (containing 2.5% linoleic acid) (Harlan Teklad, Madison, WI) or the basal diet containing either 0.5% added Menhaden fish oil with 25-30% omega-3 fatty acids as triglycerides (Sigma Chemical Co., St. Louis, MO) or 0.5% added CLA. All fatty acids were mixed into the basal diet following the protocol of Frische and Johnston (10) to minimize autooxidation. At the end of 15 consecutive feeding days, mice were weighed and i.p. injected with lipopolysaccharide (E. Coli 055:B5, Sigma Chemical Co., St. Louis, MO) at 1 mg/kg body weight in sterile HEPES buffer (Sigma Chemical Co., St. Louis, MO) or HEPES buffer alone. All mice were weighed 3, 8, 24, 48 and 72 hours post injection, and feed intake was determined at 3, 8 and 24 hours post injection.

At sacrifice, spleens were excised and single cell suspensions were made by teasing the spleen apart in calcium and magnesium free buffer (pH 7.0) and passing through an 18-gauge needle. Cells were then centrifuged through Histopaque 1077 (density 1.077) (Sigma Chemical Co., St. Louis, MO) and the buffy layer containing mononuclear cells was collected. Mononuclear cells were counted on a hemocytometer and tested for viability using trypan blue exclusion. Blastogenesis was measured following the procedure of Hughes et al (11). Results are expressed as a DPM index (disintegrations per minute in stimulated sample cells/ DPM in unstimulated sample cells).

Rat and chick experimental protocol: CLA (0.5% mixed into a semi-purified rat diet (Harlan Teklad, Madison, WI) or a standard chick mash) was fed to 20 rats (Harlan Sprague Dawley, Madison, WI) and 24 chicks (Leghorn X New Hampshire, Poultry Research Laboratory, Madison, WI) for 14 and 7 consecutive days, respectively. Control animals were fed their respective basal diets. After the feeding period, animals were injected i.p. with

endotoxin (see mouse protocol) and body weight losses due to the injection were monitored.

**Statistics:** Data were subjected to analysis of variance using the general linear models procedure of the Statistical Analysis System (12). Mean differences were determined using the least significant difference method.

### Results

Feeding CLA to mice prevented the severe body weight loss caused by endotoxin injection as observed in mice fed the basal diet (figure 1). Fish oil (.5% of diet) was ineffective in preventing endotoxin-induced weight loss. Within the first 12 hours after endotoxin injection, body weight loss was equal among treatment groups. However, by 24 hours after injection, body weight losses in basal and fish oil fed mice were twice that of CLA fed mice. Return to pre-injection body weight also appeared to be faster in the groups fed CLA. In a similar manner, twenty-four hours post endotoxin injection, the reduction in body weight gain in CLA fed chicks (+1 g/24 hrs vs. +3 g/24 hrs for noninjected controls) and rats (-8 g/24 hrs vs +9 g/24 hrs for noninjected controls) was significantly less than endotoxin injected chicks (-6 g/24 hrs) and rats (-15 g/24 hrs) fed their respective basal diets.

Mice injected with buffer consumed significantly more feed at all time periods measured than mice fed either the basal diet or basal diet plus fish oil and injected with endotoxin (figure 2). The latter two groups consumed no feed during the 24 hour period after injection. However, mice fed CLA also consumed significantly more feed than mice fed fish oil or the basal diet respectively. Mice from all treatments which were injected with

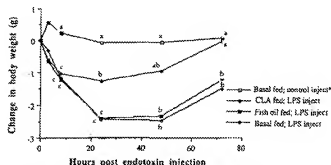


Figure 1. Change in body weight due to endotoxin injection in mice consuming diets containing CLA or fish oil.

\*Not different from CLA or fish oil fed and control inject.

abc Means with different letters are significantly different ( $p < .05$ ).

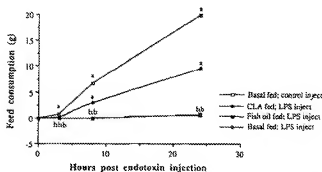


Figure 2. Effect of endotoxin injection on cumulative feed intake in mice.

ab Means with different letters are significantly different ( $p < .05$ ).

buffer had feed consumptions and body weight gains which did not differ.

Mice fed CLA had a 1.5 fold increase in PHA-P induced spleen lymphocyte blastogenesis over basal fed mice (figure 3). CLA fed mice also displayed greater responses than fish oil fed mice, although this difference was not significant.

The muscle content of CLA was increased in rats (6.25 vs. .94 mg/g fat;  $p < .05$ ) and chicks (4.07 vs. not detectable;  $p < .05$ ) fed CLA relative to basal fed controls. Muscle arachidonic acid levels were decreased in CLA fed animals relative to the basal fed control animals (60.8 vs. 39.9 mg/g fat in rats and 43.6 vs. 38.8 mg/g fat in chicks).

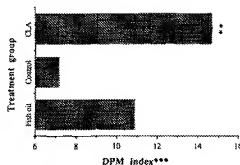


Figure 3. Effect of feeding fish oil or CLA on PHA-P induced spleen lymphocyte blastogenesis in mice.

\*PHA-P level = .78μg/500,000 cells.

\*\*Significantly greater than control ( $p < .08$ ).

\*\*\*DPM sample/DPM control.

### Discussion

Klasing et al (13) showed that endotoxin induced weight loss was the result of not only reduced food intake, but also reduced fractional protein synthesis rate in the gastrocnemius and increased skeletal muscle degradation. Likewise, the ability of animals fed CLA to maintain body weight gain during endotoxin challenge may be due to factors other than maintenance of feed intake. Endotoxin injection has been shown to enhance IL-1 production, and semi-purified IL-1 was capable of decreasing rate of gain and feed intake to the same extent as endotoxin injection (13). Recently it has been demonstrated that feed intake depression associated with immune stimulation via IL-1 injection can be prevented by feeding 8% fish oil containing omega-3 fatty acids (3). Fish oils added in human diets increased cell membrane EPA (an n-3 fatty acid) and decreased cell membrane arachidonic acid (an n-6 fatty acid). The change in cell membrane fatty acids was thought to alter prostaglandin production and thus prevented body weight loss due to IL-1 injection (3). It was also thought that a depression of PGE<sub>2</sub> at the skeletal muscle level depressed protein degradation during IL-1 stimulation (14) since IL-1 was shown to enhance muscle catabolism by inducing PGE<sub>2</sub> production (15). It is possible that CLA acted through a similar mechanism and thereby altered cell membrane fatty acids and depressed PGE<sub>2</sub> production. Indeed CLA decreased skeletal muscle arachidonic acid levels. Thus animals maintained growth during immune stimulation.

Michel et al (16) recently reported that CLA stimulated mitogen-induced lymphocyte blastogenesis, cytotoxic activity and macrophage killing ability. In agreement with Michel and co-workers, we also have shown that CLA enhances PHA-P induced lymphocyte blastogenesis relative to basal fed controls.

### Acknowledgment

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# Dietary Conjugated Linoleic Acid Decreased Cachexia, Macrophage Tumor Necrosis Factor- $\alpha$ Production, and Modifies Splenocyte Cytokines Production<sup>1</sup>

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The effect of conjugated linoleic acid (CLA) on macrophage functions were studied *in vitro*, *in vivo*, and *ex vivo*. In RAW macrophage cell line, CLA (mixed isomers) was shown to inhibit lipopolysaccharide (LPS)-stimulated tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) production. Two CLA isomers, c9,t11 and t10,c12, were tested on RAW cells and it was found that the c9,t11 was the isomer responsible for the inhibition of LPS-induced TNF- $\alpha$  production. BALB/c mice were used to determine the effect of dietary CLA on body weight wasting and feed intake after LPS injection. CLA was protective against LPS-induced body weight wasting and anorexia. Plasma TNF- $\alpha$  levels after LPS injection were lower in the CLA group compared with the corn oil-fed control group 2 hr post-LPS injection. In a separate experiment, 30 mice were fed a CLA-supplemented diet or a corn oil-supplemented diet for 6 weeks and peritoneal resident macrophages were obtained for measuring TNF- $\alpha$  and nitric oxide production after *in vitro* exposure to interferon- $\gamma$  (IFN- $\gamma$ ) and/or LPS. TNF- $\alpha$  production was not found to be different in peritoneal macrophages from mice fed the dietary treatments, but less nitric oxide was produced in macrophages from CLA-fed mice upon stimulation when compared with macrophages from control-fed mice. Splenocytes were also collected from the mice fed the dietary treatments and stimulated to produce cytokines in culture. Supernatant was used to run cytokine enzyme-linked immunosorbent assays. Interleukin-4 (IL-4) was decreased in CLA-fed mice when splenocytes were stimulated with concanavalin A (Con A) for 44 hr; however, IL-2 and the IL-2-to-IL-4 ratio were elevated. *Exp Biol Med* 228:51–58, 2003

**Key words:** CLA; cachexia; macrophage; cytokines

A biological important role of conjugated linoleic acid (CLA) as an anticarcinogen was first reported by Pariza and Hargraves (1). It was later found that CLA had physiological properties that differed from LA. Synthetic CLA is a mixture of isomers, and it is difficult and costly to purify pure isomers, hence, until recently, most reports on the physiological effects of CLA have used mixed isomers (predominantly c9,t11 and t10,c12). Among these studies, CLA has been shown to decrease carcinogenesis (2, 3), decrease atherosclerosis (4, 5), and change body composition by increasing body protein and water content and decreasing body fat (6–8). In addition to the above-mentioned properties, CLA also modulates the immune system by increasing lymphocyte blastogenesis, lymphocyte cytotoxic activity, and macrophage killing ability, as well as protecting against end-stage body wasting in autoimmune disease (9–12). Other fatty acids also affect immunity as well (13–15).

It has been shown that several dietary oils modulate macrophage function. For example, dietary fish oil decreased interleukin-1 (IL-1) release by peritoneal macrophages when compared with corn oil-fed controls (16). Macrophages are believed to be the principal sources of tumor necrosis factor (TNF)- $\alpha$  produced *in vivo*, and lipopolysaccharide (LPS) is the most potent stimulus of macrophages for TNF- $\alpha$  production. The activity of TNF- $\alpha$  was originally shown to kill tumor cells, but it also has a profound effect in causing body wasting, or cachexia. Direct infusion of TNF- $\alpha$  into rats has been shown to promote muscle degradation (17). Our laboratory has shown that feeding CLA to chicks reduces LPS-induced body weight wasting and feed intake (12, 18). Because macrophages are immune cells known to produce TNF- $\alpha$  upon LPS stimulation, the effect of CLA at inhibiting LPS-induced wasting could be due to decreased TNF- $\alpha$  production by macrophages.

T lymphocytes play an important role in the immune system. Upon activation, naive T cells (Th0) differentiate into either T helper 1 (Th1) or Th2 effector cells as they secrete different cytokines and mediate very different im-

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mune responses (19). Th1 type responses are primarily cell-mediated immunity and inflammation, and Th2 cells and cytokines mediate humoral immunity. Upon activation, development of Th0 cells into either Th1 or Th2 cells depends on the local cytokine milieu. For example, in the presence of IL-12, Th1 clone development is preferred (20, 21). On the other hand, IL-4 is indispensable for Th0 cells to polarize into Th2 clones (22, 23). Moreover, Th1 and Th2 responses are cross-regulated. For example, IFN- $\gamma$ , a cytokine produced by Th1 cells, inhibits IL-4 production and suppresses Th2 development (24, 25). Conversely, IL-4 and IL-10 produced by Th2 cells block differentiation of Th0 to Th1 (26, 27). Sugano *et al.* (11) showed dietary CLA increased immunoglobulin (Ig) A, IgG, and IgM in both rat serum and cultured lymph node cells, whereas IgE was reduced. This work implied that CLA may promote Th1 cytokine and inhibit Th2 cytokine production, as Ig class switch from IgG to IgE would not occur without IL-4 or IL-13, both which are potent Th2 cytokines (28, 29). In a guinea pig asthma model, allergen-induced trachea histamine production was reduced in CLA-fed guinea pigs (30). IgE-induced histamine release was enhanced by IL-5, a Th2 cytokine (31). It was our interest to test the effect of CLA on the helper T cell cytokines profile modification as suggested by previous studies.

Because macrophages are very sensitive to LPS stimulation, it seemed that macrophages can be a potential target of CLA to decrease LPS-induced responses. In this study, we investigated the effect of CLA on macrophage TNF- $\alpha$  production, as it was hypothesized that CLA inhibits TNF- $\alpha$  production by macrophages. In addition, splenocyte cytokines IL-2 and IL-4 were also measured.

## Material and Methods

**CLA.** CLA used in the *in vivo* and *ex vivo* feeding trials was obtained from Natural Lipids Inc. (Hovdebygd, Norway) and contained approximately 90% CLA (CLA-90) with the following C18:2 conjugated isomer distribution: 43.5% t10,c12; 41.9% c9,t11 and t9,c11; 1.5% t9,t11 and t10,t12; 0.9% c9,c11; and 0.9% c10,c12. Other fatty acids in CLA-90 were 5.6% oleate, 1.4% palmitate, 0.5% linoleate, 0.4% stearate, and 3.4% unidentified compounds. For *in vitro* studies, LA was purchased from Nu-Check Prep (>99% pure; Elysian, MN). The c9,t11 CLA isomer was purchased from Matreya Inc. (Pleasant Gap, PA). The c9,t11 CLA isomer was 96.3% pure and had 2.6% of t9,t11/t10,t12 and 1.1% of other CLA isomers. The t10,c12 isomer was from Natural Lipids, and it had 92.8% of t10,c12; 1.6% of c9,t11; 1.2% of t9,t11/t10,t12; and 2.8% of other CLA isomers.

**Macrophages.** RAW 264.7 cells were a gift from M.W. Pariza (Food Research Institute, University of Wisconsin, Madison, WI). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) media (Life Technologies, Gaithersburg, MD) supplemented with 5% fetal bovine serum (FBS) and 1% antibiotics solution (A9909; Sigma,

St. Louis, MO). Macrophages were plated in the density of 100,000 cells/well in a 24-well plate for 24 hr, followed by culture in fresh media containing fatty acid-albumin complex. The final fatty acid concentration was 100  $\mu$ M. Cells were cultured with fatty acid-albumin complex for 24 hr. Fresh media (containing corresponding fatty acid-albumin complex) containing 500 ng/ml LPS (from *E. coli*, serotype 055:B5; L-4005; Sigma) in phosphorus-buffered saline (PBS) was then added for 16 hr to stimulate TNF- $\alpha$  secretion. Control cells were treated with PBS. Media was then collected, frozen, and subsequently analyzed for TNF- $\alpha$  as described below. Cell viability was checked under microscope and by total nonwashable protein measurement (cell protein concentration was measured after removing supernatants for TNF- $\alpha$  analysis and washing cells with PBS three times) to confirm that viability of macrophages cultured under the 100  $\mu$ M fatty acid-albumin complex did not differ from macrophages cultured with albumin alone.

**Preparation of Fatty Acid-Albumin Complex.** To make fatty acid-albumin complexes for treating macrophages *in vitro*, 2.8 mg of free fatty acid, already dissolved in 0.5 ml of KOH (0.1 M), was transferred to a scintillation vial, and 4 ml of 2.5 ml bovine albumin PBS solution (LPS free, A-8806; Sigma) was added and gassed with nitrogen. The vial was then wrapped with foil and refrigerated overnight. The pH was then adjusted to 7.2 using 0.1 M NaOH solution, and the volume was brought to 5 ml with PBS. The solution was filtered using a 0.22- $\mu$ m syringe filter for use in cell culture. The fatty acid concentration of the preparation was 2 mM.

**Diet.** A semipurified powdered diet was purchased from Harlan-Teklad (TD94060, 99% basal mix; Madison, WI). The diet had 5% corn oil and it was supplemented with either 0.5% CLA or corn oil and 0.5% sugar. Hence, the control diet had 5.5% corn oil, and the CLA diet had 5% corn oil and 0.5% CLA (10). Fresh diets were prepared every other week and were kept refrigerated. Fresh diet was provided to mice three times a week. Both diets and water were provided *ad libitum*.

**Animals.** Weanling BALB/c mice were purchased from Harlan-Sprague Dawley (Indianapolis, IN). In experiment one, 12 mice were immediately divided into two groups of six and were fed either CLA or the control diet. Three mice were housed together in a shoebox cage during dietary treatment and were then individually caged right before LPS injection (see below). In the *ex vivo* study, 30 mice were used in the study with 15 mice who were fed the control diet and 15 mice who were fed the CLA diet. Three mice were housed per cage in a temperature- and humidity-controlled room with a 12:12-hr light:dark cycle. The protocol for animal care and use was approved by the institutional animal care and use committee at the College of Agriculture and Life Sciences, University of Wisconsin-Madison.

**In Vivo Mouse Trial Treatment.** After 6 weeks on the dietary treatments, three mice from each dietary group

were injected intraperitoneally with LPS (0.1 mg/ml in sterile PBS, 1 ml LPS solution/100 g of body weight) and the remaining three were injected with sterile PBS (injection control). Body weight and feed intake were recorded at 0, 24, 48, and 72 hr postendotoxin injection on the individually housed mice. Blood samples were obtained retro-orbitally at 0, 1, and 2 hr after LPS injection (32). Plasma samples were taken from the blood samples after centrifugation and were subsequently analyzed for TNF- $\alpha$  as described below.

**Isolation and Culture of Mouse Resident Peritoneal Macrophages.** In the *ex vivo* study, 30 mice were either fed the CLA (15 mice) or control diet (15 mice) for 6 weeks. Two or four mice were then sacrificed daily to obtain resident peritoneal macrophages and splenocytes. Peritoneal resident macrophages were collected post-euthanasia by injecting 10 ml of ice-cold DMEM media containing 10% FBS and 1% of antibiotics solution (Sigma) into the peritoneal cavities, and then recovering the fluid (33). The fluid was collected and gently laid on top of 3 ml of Histopaque 1081 (Sigma) and was centrifuged at 400g for 15 min at ambient temperature. Mononuclear cells were transferred to a clean centrifuge tube and were washed twice in  $Mg^{2+}$ - $Ca^{2+}$ -free PBS. Viable cell numbers, as determined by trypan blue exclusion, were greater than 95%. The cells were suspended and adjusted to  $1 \times 10^6$  viable cells/ml in RPMI 1640 medium with 10% FBS and 1% antibiotics solution (Sigma). One hundred microliters of cell suspension was added to individual wells in a 96-well plate (100,000 cells/well) for 2 hr. Nonadherent cells were removed by washing the monolayer twice with fresh medium. The adherent peritoneal exudate cells are hereafter referred to as macrophages. Macrophages were immediately primed with 5 unit/ml IFN- $\gamma$  for 4 hr before being stimulated for TNF- $\alpha$  or nitrite production (33). Macrophages were cultured with LPS (500 ng/ml) for 16 hr and the supernatants were collected for TNF- $\alpha$  assay. For nitric oxide assay, phenol-red free DMEM was used, and 500 ng/ml LPS and 5 unit/ml IFN- $\gamma$  were added to the macrophages and incubated for 44 hr before the culture supernatants were removed for nitrite assay (33).

**Splenocyte Isolation.** Spleens were removed from the CLA- or control-fed mice and were placed in a petri dish with 10 ml of RPMI 1640 media. A 10-ml sterile syringe plunger was used to disperse the spleen into a single-celled suspension. The media containing the suspended splenocytes were then collected and layered atop 3 ml of Histopaque 1081 (Sigma) in a 15-ml centrifuge tube and were centrifuged at 400g for 15 min at ambient temperature. Mononuclear cells at the interface were collected using a transfer pipette, placed in another centrifugation tube, and 10 ml of media was added. Cells were centrifuged and the pellet was then washed twice with fresh media by centrifugation. Splenocytes were then resuspended to a cell density of  $2 \times 10^6$ /ml, and 500  $\mu$ l was applied into each well in a 24-well plate. Concanavalin A (Con A) at 4  $\mu$ g/ml was added into splenocyte culture, and supernatant was col-

lected after 48 hr and frozen for subsequent cytokine analysis.

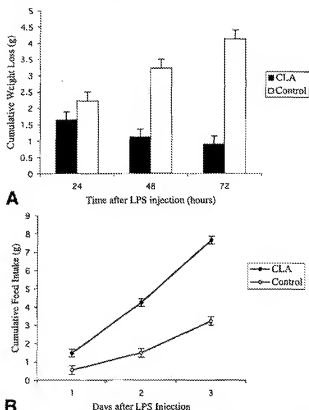
**Cytokine Assay.** TNF- $\alpha$ , IL-2, and IL-4. OptEIA enzyme-linked immunosorbent assay (ELISA) kits (PharMingen, San Diego, CA) were used to determine the serum TNF- $\alpha$  level as well as culture supernatant IL-2, IL-4, and TNF- $\alpha$  concentration. A standard procedure of cytokine ELISA was performed according to the manufacturer's instructions. Briefly, a plastic plate was coated overnight with a capture antibody for a specific cytokine, followed by washing and blocking the plate. Diluted samples and standards (provided in the kit) were then added and incubated. An extensive wash was applied before secondary antibody and enzyme conjugates were added. Plates then went through another round of incubation and extensive washing. Developing reagent was then added to the plate for 15 min. Color development was stopped during linear increases in substrate utilization by adding 0.5 M sulfuric acid to disrupt enzymatic activity. OD readings of samples were converted to concentration based on the reference curve. At least duplicate samples were analyzed for each cytokine ELISA. The TNF- $\alpha$  kit used in the present study only detects free-form TNF- $\alpha$  but not receptor-bound form. The detection limit for TNF- $\alpha$ , IL-2, and IL-4 is 20, 10, and 10 pg/ml, respectively.

**Quantification of Nitrite in Culture Supernatant.** Measurement of nitrite has been commonly used as an indirect measurement of nitric oxide formation because nitrite is a stable end product of the highly reactive nitric oxide (34). In the *ex vivo* experiment, 50  $\mu$ l of cultured resident macrophage supernatants was taken from each well and mixed with 50  $\mu$ l of Griess reagent [1 part of 1% sulfanilamide and 1 part of 0.1% N-(1-naphthyl) ethylenediaminedihydrochloride, mixed immediately before use] in a different 96-well plate. The plate was gently shaken at ambient temperature for 5 min, and OD was measured at 562 nm. A standard curve was established using known  $NaNO_2$  preparations. Sample nitrite concentration was extrapolated from the OD based on the standard curve. Duplicate samples were used in every nitrite microplate assay. The typical standard curve had a correlation coefficient of more than 99% between 0 and 20  $\mu$ M. Samples were diluted to this range and were analyzed again if they were found to be higher than the upper limit of standards at first analysis.

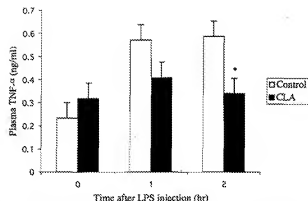
**Colorimetric Tetrazolium Assay.** The linear relationship between number of macrophages or splenocytes and cleavage of tetrazolium was measured by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) assay (35). MTT was dissolved in PBS (5 mg/ml) and filter sterilized through a 0.22- $\mu$ m syringe filter. Twenty-five microliters of MTT solution was added to each culture well and these plates were incubated at 37°C for 3 hr. One hundred microliters stop reagent was added to each well and mixed. Stop reagent was prepared by dissolving sodium dodecyl sulfate 20% (w/v) at ambient temperature in 50% N,N-dimethylformamide in demineralized wa-

ter, and pH was adjusted to 4.7 using 80% acetic acid and 2.5% 1 N HCl. The plates were incubated overnight at 37°C to ensure the complete solubilization of cells and the blue crystals of formazan. OD was read on a microplate reader at wavelength of 562 nm. MTT values were determined after Con A stimulation and were used to correct IL-2 and IL-4 data based on cell numbers.

**Statistical Analysis.** Both nonrepeated and repeated data were analyzed to determine CLA treatment effect by PROC MIX in SAS computer program, version 8 (SAS Institute, Cary, NC) (36). For Figures 1 and 2, data were analyzed by one-way analysis of variance (ANOVA) with two treatments, with repeated measures on experiment unit (mouse). A type "ARH(1)" error structure was used to account for auto-correlated errors. For Figures 3 and 4, data were analyzed by one-way ANOVA with diet as treatment and mice(diet) as error. In addition, the model included a blocking factor to account for variations due to different days of animal sacrifice. For Figure 5, data were analyzed by randomized complete block design with experiment unit



**Figure 1.** The influence of dietary CLA on LPS-induced body weight loss and feed intake. After feeding CLA or control diet for 6 weeks, mice were injected with either LPS (1 mg/kg) or PBS (data not shown). Cumulative body weight change and feed intake were monitored for 3 days. Each point represents least square mean with pooled error. There were three mice in each group. (a) Repeated data analysis showed dietary CLA was protecting mice against weight loss compared with mice fed the control (corn oil) diet ( $P < 0.01$ ). (b) CLA-fed mice ate more diet after LPS injection compared with control-fed mice ( $P < 0.01$ ).



**Figure 2.** Influence of dietary CLA on LPS-induced release of plasma TNF- $\alpha$ . Mice were fed a CLA or control diet for 6 weeks before LPS (1 ng/g body weight) or PBS injection. Blood samples were obtained from mice 0, 1, and 2 hr after LPS injection. ELISA was used to determine plasma TNF- $\alpha$  level. Each bar represents least square mean + pooled error (SEM). Plasma TNF- $\alpha$  was unchanged over time in the PBS-injected mice (data not shown). An asterisk indicates significantly different from control at  $P < 0.01$ .

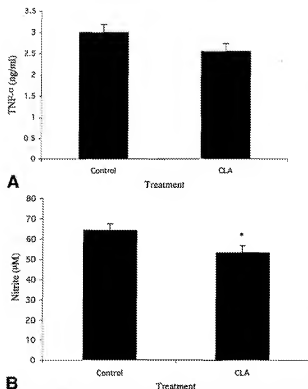
was mean of quadruplicate wells. Data from five independent experiments were used in the analysis where experiment was treated as block.

## Results

Mice fed CLA had significantly less endotoxin-induced body weight loss (0.9 g) over 3 days compared with control-fed endotoxin-injected mice (4.1 g; Fig. 1A). Feed intake after LPS injection was also significantly affected by CLA. After LPS injection, CLA-fed mice ate more feed (2.6 g/day) compared with control-fed mice (1.1 g/day) over a 3-day period (Fig. 1B). In PBS injection treatment, feed intake was 2.8 g/d for the CLA group and 2.6 g/d for the control group. Weight loss in PBS injection treatment was 1.7 g in the control group and 0.1 g in the CLA group (data not shown). In the *in vivo* trial, CLA had a potent inhibitory effect on LPS-induced anorexia and body weight loss.

The evidence that CLA protects against LPS-induced cachexia and the role of dietary CLA on LPS-induced plasma TNF- $\alpha$  was investigated. After mice were fed CLA for 6 weeks, the plasma TNF- $\alpha$  levels were significantly lower in the CLA-fed mice compared with the control-fed mice 2 hr after LPS injection (Fig. 2). The TNF- $\alpha$  production from PBS injection control was unchanged (data not shown).

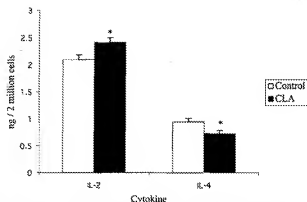
In the *ex vivo* trial, we studied peritoneal resident macrophage activity after mice were fed the dietary treatments for 6 weeks. We found that even though CLA decreased plasma TNF- $\alpha$  level *in vivo*, CLA did not statistically decrease LPS-induced release of TNF- $\alpha$  in peritoneal resident macrophage in this *ex vivo* system (Fig. 3A). Nitric oxide production, however, was reduced by CLA. Peritoneal resident macrophages from CLA-fed mice had less nitric oxide production when stimulated by IFN- $\gamma$  and LPS when compared with cells from control-fed mice (Fig. 3B).



**Figure 3.** Nitric oxide and TNF- $\alpha$  production by resident peritoneal macrophages were affected by CLA feeding. Mice were fed CLA or control diet for 6 weeks before sacrifice. Resident peritoneal macrophages were obtained and primed with IFN- $\gamma$ . (a) Macrophages were later stimulated with LPS for TNF- $\alpha$  production. (b) Macrophages were later stimulated with LPS and IFN- $\gamma$  for nitric oxide production. Macrophages from mice fed a CLA diet produced a significantly lower amount of nitrite (53.3  $\mu$ M) compared with macrophages from mice fed a control diet (64.2  $\mu$ M). TNF- $\alpha$  production, however, was not different between CLA and control groups. Each bar represents least square mean with pooled error. There were 15 mice in each diet treatment. An asterisk indicates significantly different from control at  $P < 0.05$ .

Spleens were also obtained for lymphocyte cytokine analysis. Splenocytes were treated with Con A for 48 hr before collecting supernatant for cytokine analysis. Splenocytes from CLA-fed mice did show a higher IL-2 production after LPS stimulation and lower IL-4 production than splenocytes from control-fed mice (Fig. 4). The ratio of IL-2 to IL-4 was significantly ( $P < 0.05$ ) higher (ratio = 3.3) for splenocytes from the CLA-fed mice compared with the control-fed mice (ratio = 2.2).

In the *in vitro* experiment, after RAW macrophages were cultured with fatty acid-albumin complexes, they were stimulated with LPS to produce TNF- $\alpha$ . Our data showed that CLA mixture had an inhibitory effect on TNF- $\alpha$  production compared with the LA control. Among isomers, the c9,t11 isomer was responsible for this inhibitory effect (Fig. 5A), but not the t10,c12 isomer (Fig. 5B). In average, 100  $\mu$ M c9,t11 CLA isomer decreased macrophage TNF- $\alpha$  production by 60% compared with LA control. This c9,t11 isomer effect was dose responsive as 50  $\mu$ M c9,t11 CLA



**Figure 4.** Cytokine production by splenocytes from mice fed CLA or control diet. After mice were fed CLA or control diet for 6 weeks, splenocytes were harvested and stimulated with concanavalin A for cytokine production. Supernatants were then harvested from the cultured splenocytes and analyzed by ELISA. Cytokine production from each well was corrected by numbers of cells presented at the end of the culture that was determined by colorimetric tetrazolium assay as described in "Materials and Methods." Each bar represents least square mean with pooled error. There are 15 mice in each diet treatment. The IL-2-to-IL-4 ratio is 3.3 for the CLA group and 2.2 for the control group. An asterisk indicates significantly different from control at  $P < 0.05$ .

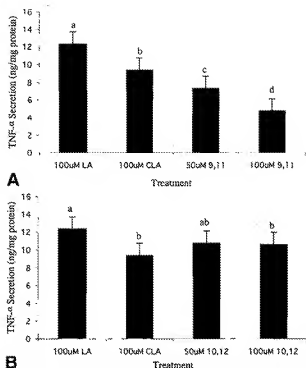
alone was 40% inhibitory to macrophage TNF- $\alpha$  production. On the other hand, t10,c12 isomer did not show any effect on macrophage TNF- $\alpha$  production.

## Discussion

Dietary fatty acids have been shown to be potent immune regulators. In general, high dietary levels of PUFA or n-3 fatty acids have an inhibitory effect on T cell proliferation and natural killer (NK) cell activity (37). Fish oil, rich in n-3 fatty acids, decreased murine and human LPS-stimulated TNF- $\alpha$  production (38, 39). It has been shown that dietary fish oil prevents endotoxin-induced death in guinea pigs (40), possibly by inhibition of cytokines produced by macrophages (41).

Feeding 0.5% CLA to animals resulted in up to 10 mg of CLA per gram of body fat in several different tissues within 30 days (42), and it took between 2 and 4 weeks to reduce CLA to baseline levels after its withdrawal from the diet (43). Several physiological changes, including body composition change (6), anticarcinogenesis (44), and atherosclerosis (4), were also shown when using up to 0.5% CLA in animal studies.

CLA has been shown to be a potent immune regulator. CLA decreased the LPS-induced cachectic response in chicken, rats, and mice, but at the same time, increased lymphocyte blastogenesis (12, 18). Other groups also reported CLA-increased lymphocyte blastogenesis as well as increased IL-2 production (9, 45, 46). To the best of our knowledge, this is the first report of an elevated Th1/Th2 cytokine ratio in CLA-fed mice. The increased IL-2 production (shown here, and by others) and the decreased IL-4 production associated with dietary CLA may explain the



**Figure 5.** CLA inhibited TNF- $\alpha$  production from cultured RAW macrophage cell line (a and b). RAW cells were plated (100,000 cells/well) in 24-well plate for 24 hr. Then they were cultured with fatty acid-albumin complex for 24 hr before LPS was added into culture to stimulate TNF- $\alpha$  secretion for 16 hr. Each bar represents least square mean with pooled error (SEM). There were 4 wells for each treatment in a 24-well plate. Bar with a different letter indicates significance difference at  $P < 0.05$ . (a) Compared among LA, CLA, and c9, t11 CLA isomer. (b) Compared among LA, CLA, and t10, c12 CLA isomer. Mixed isomers, approximately 42% c9,t11 and 44% t10,c12. Figure shown here represented means of five independent experiments.

reduced IgE production in CLA fed rats (11) as compared with rats fed diets largely based on LA.

Because CLA seems to enhance immunological responses, which may pose a potential risk on immune hypersensitivity, our laboratory has been studying how dietary CLA may affect type I and type III immune hypersensitivity. In a type I hypersensitivity model, CLA reduced antigen-induced histamine and PGE<sub>2</sub> release from trachea of CLA-fed guinea pigs (30). In an autoimmune (type III hypersensitivity) model, CLA feeding not only protected against end-stage body wasting, but also increased survival days 1.5-fold after the onset of proteinuria in mice (10). Shifted cytokine profiles toward Th1 may interfere with Ig class switch that is needed in producing IgE in immune type I hypersensitivity responses (47). The reduced IgE production by CLA-fed rats (11) would certainly result in a reduced type I hypersensitivity reaction upon challenge, as demonstrated in a guinea pig hypersensitivity model (30). The development of experimentally inducible lupus in mice seems to involve two stages: increased Th1 cytokines followed by elevated Th2 cytokines later in life (48). Increased Th2 cytokine production correlates well to disease progres-

sion. Hence, a CLA-induced shift toward Th1 cytokines could explain, at least in part, increased days of survival post-proteinuria in lupus-prone mice. Inhibited production of TNF- $\alpha$  may also help to explain the reduction in body weight loss in CLA-fed lupus mice at the end stage of systemic lupus disease (10).

Macrophages are sensitive to dietary fatty acid supplementation, as its fatty acid profile reflects such dietary intervention (49, 50). These works also showed that dietary fatty acids also dictate macrophage physiology. Dietary fish oil has been shown to decrease macrophage antigen presentation (51, 52) and to decrease cytokine production as well as mRNA expression (38, 53). In the present study, macrophages were tested as one of the target cells on which CLA modulates immune function. Our data showed that CLA, more specifically c9,t11 CLA, decreased TNF- $\alpha$  production in RAW macrophage cell line. LPS-induced anorexia and cachexia responses were reduced in CLA-fed animals. The plasma TNF- $\alpha$  level after LPS injection was also suppressed in CLA-fed mice. It was unanticipated that mice fed CLA did not suppress *ex vivo* TNF- $\alpha$  production in resident peritoneal macrophages. However, both *in vitro* and plasma samples showed that CLA has an inhibitory effect on TNF- $\alpha$  production. Fatty acid turnover rate in cultured peritoneal resident macrophages may have played a role in the lack of TNF- $\alpha$  response observed in this model. In the cell culture study, CLA was kept at a constant concentration during the *in vitro* experiments, and when blood was drawn for TNF- $\alpha$  analysis, mice were still fed the treatment diet. However, when culture supernatants were collected in the *ex vivo* study, peritoneal macrophages had already been cultured *in vitro* for 24 hr without exogenous exposure to CLA. Moreover, the fetal bovine serum used in the system might not have been as effective as autologous serum (15) in demonstrating CLA's effects on TNF- $\alpha$  production by resident peritoneal macrophages.

The two main isomers of the CLA mixture (c9,t11 and t10,c12) used in this study were considered to have different properties on cytokine regulation. In fact, c9,t11 CLA increased feed efficiency (54, 55), whereas the t10,c12 was the isomer shown to affect body composition (56). In the present study, c9,t11 CLA isomer inhibited TNF- $\alpha$  production from *in vitro* macrophage cultures (Fig. 5a). The slight decrease of TNF- $\alpha$  in 100  $\mu$ M t10,c12 CLA isomer treatment probably was thought to be due to c9,t11 CLA isomer contamination of the t10,t12 CLA isomer used (Fig. 5b). Moreover, a recent study reported decreased hepatic TNF- $\alpha$  mRNA expression by c9,t11 CLA in mice (57). It remains to be shown which isomers downregulate IL-4 production and increase IL-2 production. Even though the *in vitro* data suggested that c9,t11 CLA is the active isomer in reducing LPS-induced TNF- $\alpha$  production, further *in vivo* studies with pure CLA isomers are required to validate the *in vitro* data on TNF- $\alpha$  production and determine the effects of different CLA isomers on immune function.

TNF- $\alpha$  was shown to be involved in endotoxin-induced

weight loss and cancer cachexia (58). Muscle degradation was enhanced by TNF- $\alpha$ . Blocking TNF- $\alpha$  function by either injection of anti-TNF- $\alpha$  antibody or TNF- $\alpha$ -binding proteins reduced the cachexia reaction (59, 60). The decreased LPS-induced TNF- $\alpha$  production by CLA may provide at least one mechanism by which CLA enhanced the rate of growth and improved feed efficiency in animals (12, 54, 55).

Thromboxane A<sub>2</sub> (TXA<sub>2</sub>) is a mediator of renal damage (61). In murine lupus, TXA<sub>2</sub> inhibitor prolonged survival of NZB/W F1 mice, and renal TXA<sub>2</sub> production was elevated in F1 mice (62). In a study comparing TXB<sub>2</sub> (stable end product of TXA<sub>2</sub>) production and COX gene expression of peripheral blood mononuclear cells (PBMC) among patients with active or inactive lupus nephritis and healthy individuals as a control, COX-2 expression and TXB<sub>2</sub> production in PBMC was only elevated in patients with active lupus nephritis (63). Immunostaining of kidney biopsies showed no difference in COX-1, but COX-2 staining in patients with active lupus nephritis was increased compared with patients with inactive lupus nephritis or healthy controls. Double staining of kidney biopsies with anti-COX-2 and anti-CD68 antibodies (macrophage marker) demonstrated that upregulated COX-2 enzyme expression was within the macrophages of glomeruli in patients with active lupus nephritis (63).

Infiltrating macrophages in glomeruli, with upregulated COX-2 enzyme expression, may contribute to the elevated TXB<sub>2</sub> level in active lupus nephritis. Regulation of macrophage activities and eicosanoid profiles by CLA may play a role in lupus nephritis. Because COX-2 is the inducible form and the COX-1 is constitutively expressed, decreased antigen-induced TXB<sub>2</sub> production but not the basal level of TXB<sub>2</sub> production in CLA-fed guinea pigs provides an explanation that CLA may preferentially inhibit COX-2 activity (64). Decreased macrophage activities including TNF- $\alpha$  and nitric oxide productions seen in this study may help to explain the effect of CLA in prolonging survival of NZB/W F1 mice.

In conclusion, these data suggest that cytokine regulation by CLA could be responsible for previous reports demonstrating CLA's anticachectic effects, increased lymphocyte blastogenesis, decreased immune type-1 hypersensitivity, decreased IgE production, and prolonged life post proteinuria in the NZB/W F1 autoimmune mouse.

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## Rapid report

# NF- $\kappa$ B independent inhibition of lipopolysaccharide-induced cyclooxygenase by a conjugated linoleic acid cognate, conjugated nonadecadienoic acid

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## Abstract

10t, 12c-CLA was shown to inhibit COX-2 expression through the NF- $\kappa$ B pathway. In the current study, conjugated nonadecadienoic acid (CNA) was shown to decrease inducible COX-2 protein and mRNA and PGE<sub>2</sub> release to the similar extent as 10t, 12c-CLA in Raw264.7 macrophage. However, unlike 10t, 12c-CLA, inhibition of COX-2 mRNA/protein by CNA was independent of the NF- $\kappa$ B pathway. The data indicate the regulation of COX-2 by select conjugated fatty acids and hence their anti-inflammatory actions could operate through different signal transduction pathways.

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**Keywords:** Conjugated linoleic acid; Conjugated nonadecadienoic acid; Cyclooxygenase; NF- $\kappa$ B; Prostaglandin

Conjugated linoleic acid (CLA) has been shown to prevent adverse events associated with inflammatory diseases. In animal feeding trials, CLA (mixed isomers of 9c, 11t and 10t, 12c-CLA) was shown to decrease immune-induced wasting [1–3], to decrease inflammatory mediator release from antigen challenged airway [4,5], to increase longevity in a lupus

erythematosus model [6,7], and to decrease mucosal thickening/erosions in a model of colitis [8], and to decrease joint swelling in a monoclonal antibody-induced arthritis (Butz and Cook, unpublished data). Regulation of cyclooxygenase-2 (COX-2) [5,9], tumor necrosis factor alpha (TNF) [3,10], and inducible nitric oxide synthase [10] by CLA suggest that CLA may play a key role in inhibiting the nuclear factor kappa B (NF- $\kappa$ B) activation. Recent evidence in immune related cells and tissues suggested that CLA-induced down regulation of COX-2 protein and mRNA level may be related to an upstream regulation of signal transduction through the NF- $\kappa$ B pathway [9]. The isomer shown to inhibit the p50 and p65 subunits binding to DNA was 10t, 12c-CLA.

Structure/function studies on CLA cognates and derivatives [11] showed that a 19-carbon CLA cognate, conjugated nonadecadienoic acid (98.6% CNA, with 10c, 12c-CNA, 42.3%; 11t, 13c-CNA, 48.0%; 10c, 12c-CNA and 11c, 13c-

**Abbreviations:** CLA, conjugated linoleic acid; COX-2, cyclooxygenase-2; CNA, conjugated nonadecadienoic acid; DMEM, Dulbecco's Modified Eagle's Medium; ELA, enzyme immunoassay; ERK, extracellular signal-related kinase; FBS, fetal bovine serum; G3PDI, glyceraldehyde-3-phosphate dehydrogenase; IsBx, inhibitory  $\kappa$ B protein  $\alpha$  IKK, inhibitor- $\kappa$ B kinase; IKK, C-jun NH2-terminal kinase; LA, linoleic acid; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF- $\kappa$ B, nuclear factor  $\kappa$ B; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; PVDF, polyvinylidene difluoride; RIPA, radioimmunoprecipitation assay; TNF, tumor necrosis factor  $\alpha$ .

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CNA 3.2%, 10t, 12t-CNA and 11t, 13t-CNA 5.1%; and unidentified 1.4%, see reference [2] for the synthesis of CNA), was at least as effective as CLA in regulating fat metabolism in 3T3-L1 adipocytes or growing mice [12]. Both the 10t, 12c-CLA and CNA were shown to similarly reduce leptin secretion [13], and the mRNA and protein level of select adipocyte genes in 3T3-L1 adipocytes [14]. In our continued studies on the mechanisms by which CLA regulates COX-2, we investigated whether the new CLA cognate, CNA, also inhibits COX-2 by a similar mechanism to 10t, 12c-CLA.

We first examined whether CNA could inhibit LPS-induced COX-2 protein expression. Raw264.7 macrophage was incubated with fatty acids for 24 h and then stimulated with LPS (100 ng/ml) for 8 h in the presence of LA, 10t, 12c-CLA or CNA (0, 3.3, 10, 33 and 100  $\mu$ M). COX-2 was not induced without LPS stimulation. COX-2 protein level was significantly inhibited by CNA at 33  $\mu$ M and 100  $\mu$ M (77.8% and 58.7% of LPS-stimulated ethanol vehicle treated cells respectively, Fig. 1A, ethanol <0.2%). Similarly, COX-2 protein level was also reduced by 10t, 12c-CLA at 33  $\mu$ M and 100  $\mu$ M to 46.7% and 26.4% of LPS-stimulated cells (ethanol vehicle-treated only, Fig. 1A). 10t, 12c-CLA was more potent in reducing COX-2 protein expression compared with CNA (Fig. 1A). Since CNA only contains 48% 11t, 13c isomer (the hypothesized active isomer) in the mixture (the corresponding isomer of 10t, 12c-CLA in CNA), 10t, 12c-CLA and CNA appear equally effective in inhibiting COX-2 protein level.

PGE<sub>2</sub> release from macrophage was also measured to support 10t, 12c-CLA and CNA's inhibitory effects on COX-2 protein level. Basal release of PGE<sub>2</sub> from macrophage was ~1.1 ng/mg protein (without LPS exposure). CNA and 10t, 12c-CLA were both shown to inhibit PGE<sub>2</sub> release in a concentration-dependent manner. At 100  $\mu$ M, PGE<sub>2</sub> level in CNA and 10t, 12c-CLA-treated sample was reduced from 20.0 ng/mg protein (LPS stimulated/ethanol vehicle-treated only) to 5.43 ng/mg and 5.15 ng/mg (Fig. 1B). That both CNA and 10t, 12c-CLA were shown to inhibit PGE<sub>2</sub> release at their lowest concentrations suggests these isomers not only reduce protein expression but may also inhibit COX-2 enzymatic activity, as previously shown with 10t, 12c-CLA [9]. LA, as a precursor of AA, was shown to increase PGE<sub>2</sub> release into the culture media in a concentration-dependent manner [9]. PGE<sub>2</sub> release in LA-treated macrophages at 100  $\mu$ M was 31.9 ng/mg protein (Fig. 1B).

We further examined whether reduced COX-2 protein level by conjugated fatty acids could be due to the reduced mRNA level. Raw264.7 macrophage was incubated with conjugated fatty acids for 24 h and then was stimulated with LPS for 4 h in the presence of LA, 10t, 12c-CLA or CNA. It was shown that CNA reduced mRNA level significantly at 10  $\mu$ M, 33  $\mu$ M, and 100  $\mu$ M (by 48.9% at 100  $\mu$ M) while 10t, 12c-CLA reduced COX-2 mRNA at 100  $\mu$ M by 23.6% (Fig. 2). LA was shown to have no effect on COX-2 mRNA level (Fig. 2). We have previously shown differential regulation of 10t, 12c-CLA on COX-2 mRNA and protein level [9].

One pathway leading to the transcription of COX-2 after LPS stimulation involves the activation of the NF- $\kappa$ B via toll like

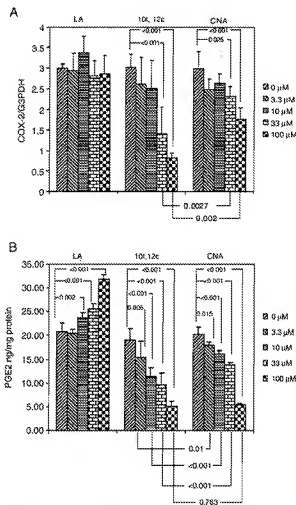


Fig. 1. Effect of CNA on LPS-induced COX-2 protein expression (A) and PGE<sub>2</sub> release in Raw264.7 macrophage.  $1 \times 10^6$  cells were seeded in each well of a 24-well plate and were grown to confluence. LA, 10t, 12c-CLA and CNA (0, 3.3, 10, 33, 100  $\mu$ M) were incubated with cells for 24 h before and during LPS (100 ng/ml) stimulation for 8 h (see reference [9]). Cell lysate and culture medium were harvested for western blot analysis of COX-2 (A) and PGE<sub>2</sub> determination by enzyme immunoassay (EIA, B). For western blot, the total protein level in the cell lysate was quantitated with Bradford assay (BioRad Laboratories, Hercules, CA) [22] and 25  $\mu$ g protein per lane was loaded onto a 10% SDS-polyacrylamide gel and resolved by electrophoresis. Proteins on the gel were transferred to polyvinylidene difluoride (PVDF) membrane. After non-specific blocking in 5% non-fat milk solution, the membrane was incubated with primary COX-2, or GAPDH antibody, then a secondary horseradish peroxidase-coupled antibody before being exposed to Fuji medical X-ray films. In panel A, ratio of COX-2/GAPDH was obtained by normalizing the band area of COX-2 to corresponding band area of GAPDH. CNA was synthesized by Dr. Ken Ku from the Food and Drug Administration, USA. The composition of CNA was 10t, 12c-CNA, 42.3%, 11t, 13c-CNA, 48.0%; 10t, 12c-CNA and 11t, 13c-CNA 3.2%; 10t, 12c-CNA and 11t, 13t-CNA 5.1%; and unidentified 1.4%. 10t, 12c-CLA (>98%) and LA (>99%) were purchased from Matreya, Inc. (Pleasant Gap, PA). COX-2 antibody and PGE<sub>2</sub> EIA kits were from Cayman Chemical (Ann Arbor, MD) and GAPDH antibody was from Trevigen (Gaithersburg, MD). These experimental results were repeatable in at least two independent experiments. Data are presented as means  $\pm$  SD from four replicates of one representative experiment. SAS procedure GLM was used to do the multiple regressions.  $P$  value <0.05 was considered statistically significant.

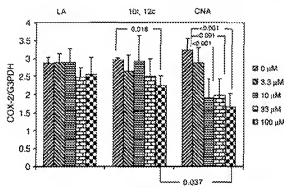


Fig. 2. Effect of CNA on LPS-induced COX-2 mRNA level in Raw264.7 macrophage.  $1 \times 10^6$  cells were seeded in each well of a 24-well plate and were grown to confluence. LA, 10t, 12c-CLA and CNA at different concentrations (0, 3.3, 10, 33, 100  $\mu$ M) were incubated with cells for 24 h before and during LPS (1000 ng/ml) stimulation for 4 h. mRNA isolation, reverse transcription and polymerase chain reaction were conducted as previously described (see reference [9]). Briefly, Raw264.7 macrophage was lysed in 100  $\mu$ l lysis buffer solution (4 M guanidium isothiocyanate, 0.5% sarcosyl, 10 mM Tris-HCl, pH 8.0 and 1% diethylenetriol) and DNA was sheared by passing cell lysate through a 25 gauge needle. Binding buffer 200  $\mu$ l (10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 400 mM NaCl) was added for mRNA to combine with magnetic particles. Supernatants from the above solution were incubated with magnetite oligo(dT) magnetic particles for at least 5 min. The particles were then washed for 4 times with wash buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) and the associated mRNA were rinsed off the beads with 10  $\mu$ l elution buffer (2 mM EDTA). Isolated mRNA (2  $\mu$ l) was reverse-transcribed in the master mix (reaction buffer 7.0  $\mu$ l, random primer 100  $\mu$ l,  $\mu$ l, dNTP 10 mM 0.4  $\mu$ l, reverse transcriptase 0.2  $\mu$ l and  $H_2O$  12.4  $\mu$ l). Polymerase chain reaction was used to amplify the individual gene using respective primers. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene was amplified as internal control. The respective primers are: COX-2 upstream: 5'-CAA-GCA-GTG-GCA-AGG-GCT-CCA-3' and downstream: 5'-GGC-ACG-TGC-ATT-GAT-GGT-GGC-3'; G3PDH upstream: 5'-GGC-ATT-CTC-GGC-TAC-ACT-GA-1 and downstream: 5'-CAT-ACC-AGG-AAA-TGA-GCT-TGA-C-3'. Ratio of COX-2/G3PDH mRNA level. These experimental results were repeatable in at least two separate experiments. Data were presented as mean  $\pm$  SD from 4 replicates of one experiment. SAS procedure GLM was used to do the multiple regressions.  $P$  value  $< 0.05$  was considered statistically significant.

receptor-4. NF- $\kappa$ B is a transcription factor which consists of heterodimer or homodimer of p50, p65, p105, p52, c-Rel or RelB. Upon stimulation by LPS, or TNF, the inhibitory proteins (I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , or I $\kappa$ B $\gamma$ ) associated with the NF- $\kappa$ B become phosphorylated and degraded in a ubiquitin-proteasome-dependent pathway [15,16]. To examine whether reduced mRNA by CNA was due to the inhibition of the NF- $\kappa$ B pathway, Raw264.7 macrophages were treated with fatty acids for 24 h and then LPS was introduced for 4 h in the presence of fatty acids. LPS increased I $\kappa$ B $\alpha$  phosphorylation as shown previously [9]. CNA was shown to have no effect on I $\kappa$ B $\alpha$  phosphorylation while 10t, 12c-CLA inhibited the I $\kappa$ B $\alpha$  phosphorylation in a concentration-dependent manner (Fig. 3). Likewise, LA had no effect on I $\kappa$ B $\alpha$  phosphorylation. Consistent with the effect of CNA, LA and 10t, 12c-CLA on I $\kappa$ B $\alpha$  phosphorylation, the increased binding of two NF- $\kappa$ B proteins (p50 and p65) to the DNA consensus sites following LPS exposure was not affected by CNA and LA, but was affected by 10t, 12c-CLA in a concentration-dependent manner (Table 1).

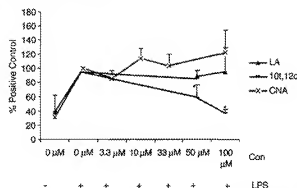


Fig. 3. Effects of CNA on phosphorylation of I $\kappa$ B $\alpha$  in Raw 264.7 macrophage.  $1 \times 10^6$  cells were seeded in each well of a 24-well plate and were grown to confluence. LA, 10t, 12c-CLA (50 and 100  $\mu$ M) and CNA (3.3, 10, 33 and 100  $\mu$ M) were incubated with cells for 24 h before and during LPS (1000 ng/ml) stimulation for 4 h. Cell lysate was harvested for western blot analysis of phosphorylated I $\kappa$ B $\alpha$  and  $\beta$ -actin. The band area of phosphorylated I $\kappa$ B $\alpha$  was normalized to corresponding band area of  $\beta$ -actin. % Positive Control = (normalized phosphorylated I $\kappa$ B $\alpha$  area in ethanol vehicle or fatty acids treated samples/normalized phosphorylated I $\kappa$ B $\alpha$  area in ethanol vehicle and LPS treated samples)  $\times 100$ . Data are presented as means  $\pm$  SD from four replicates of one experiment. Student's  $t$ -test was conducted for the statistical comparison between fatty acids, LPS treated samples and ethanol vehicle, LPS treated samples.  $P$  value  $< 0.05$  was considered statistically significant.

These data suggest that CNA regulates COX-2 by an NF- $\kappa$ B independent process and 10t, 12c-CLA may inhibit other signaling pathway(s) in addition to the NF- $\kappa$ B pathway. It is well known that stimulation of macrophage by LPS also involves activation of mitogen-activated protein kinase (MAPK) in addition to the NF- $\kappa$ B pathway [17–19]. MAPK family comprises of the extracellular signal-related kinase (ERK) 1/2, p38 MAP kinase and c-jun NH2-terminal kinase (JNK). Inhibition of individual MAPK was shown to inhibit downstream pro-inflammatory cytokine production. SB202190, a selective inhibitor of p38 MAPK, inhibited LPS-induced IL-1 $\beta$  and TNF in monocytes [19]. LPS-induced COX-2 mRNA level and PGE $_2$  release in Raw264.7 macrophage without inhibiting the NF- $\kappa$ B activity [20]. Another MAPK inhibitor, PD98059

Table 1  
Effect of CNA on nuclear factors p50 and p65 binding to the DNA consensus site

Treatments	p50	p65
Vehicle	0.384 $\pm$ 0.094 (c)	0.383 $\pm$ 0.06 (d)
Vehicle+LPS	0.728 $\pm$ 0.099 (a, b)	0.688 $\pm$ 0.11 (a)
LA + LPS	0.689 $\pm$ 0.11 (a, b)	0.599 $\pm$ 0.05 (a, b)
10t, 12c-CLA + LPS	0.556 $\pm$ 0.07 (b, c)	0.440 $\pm$ 0.06 (b, c, d)
CNA + LPS	0.664 $\pm$ 0.08 (a, b)	0.575 $\pm$ 0.09 (a, b)
LA + LPS	0.712 $\pm$ 0.08 (a, b)	0.615 $\pm$ 0.20 (a)
10t, 12c-CLA + LPS	0.548 $\pm$ 0.07 (b, c)	0.401 $\pm$ 0.02 (c, d)
CNA + LPS	0.857 $\pm$ 0.34 (a)	0.553 $\pm$ 0.07 (a, b, c)

$1 \times 10^6$  cells were seeded in each well of a 24-plate and were grown to confluence. LA, 10t, 12c-CLA and CNA at 50 or 100  $\mu$ M were incubated with cells for 24 h before and during LPS (1000 ng/ml) stimulation for 4 h. Cell lysate was harvested for the determination of p50 and p65 binding according to kit instructions (Active Motif, Carlsbad, CA). Data are presented as mean  $\pm$  SD from four replicates of one experiment. Data were analyzed by one-way ANOVA. Within each NF- $\kappa$ B subunit (p50 or p65) means without a common letter in parentheses are statistically different ( $P$  value  $< 0.05$ ).

was also shown to inhibit COX-2 mRNA and PGE<sub>2</sub> release independent of the NF- $\kappa$ B [20]. Potentially, inhibitory effects of CNA and 10 $\alpha$ , 12 $\alpha$ -CLA on LPS-induced COX-2 mRNA expression could be attributed to the inhibition of MAPK/ERK/JNK pathways, a hypothesis supported by a recent report of CLA's inhibitory effects on MAPK in osteoclast [21].

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# Structure–activity relationship of conjugated linoleic acid and its cognates in inhibiting heparin-releasable lipoprotein lipase and glycerol release from fully differentiated 3T3-L1 adipocytes

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## Abstract

Conjugated linoleic acid (CLA) reduces body fat in part by inhibiting the activity of heparin-releasable lipoprotein lipase (HR-LPL) activity in adipocytes, an effect that is induced by the *trans*-10,*cis*-12 CLA isomer. In this study we used a series of compounds that are structurally related to CLA (i.e., CLA cognates) to investigate the structural basis for this phenomenon. None of the 18:1 CLA cognates that were tested, nor *trans*-9,*cis*-12 18:2, *cis*-12-octadecen-10-ynoic acid (10y,*cis*-12) or 11-(2'-(*n*-pentyl)phenyl)-10-undecylenic acid (designated P-10), exhibited any significant effect on HR-LPL activity. Among the CLA derivatives (alcohol, amide, and chloride) that were tested, only the alcohol form inhibited HR-LPL activity, although to a lesser extent than CLA itself. In addition, intracellular TG was reduced only by *trans*-10,*cis*-12 CLA and the alcohol form of CLA. Hence it appears that the *trans*-10,*cis*-12 conjugated double bond in conjunction with a carboxyl group at C-1 is required for inhibition of HR-LPL activity, and that an alcohol group can partially substitute for the carboxyl group. We also studied glycerol release from the cells, observing that this was enhanced by *trans*-10 18:1, *trans*-13 18:1, *cis*-12 18:1, *cis*-13 18:1, P-10 but was reduced by *cis*-9 18:1, the alcohol and amide forms of CLA or 10y,*cis*-12. Accordingly the structural feature or features involved in regulating lipolysis appear to be more complex. Despite enhancing lipolysis in cultured 3T3-L1 adipocytes, *trans*-10 18:1 did not reduce body fat gain when fed to mice. © 2004 Elsevier Inc. All rights reserved.

**Keywords:** Conjugated linoleic acid; CLA; Lipoprotein lipase; 3T3-L1; *cis*-9; *trans*-11 CLA; *trans*-10; *cis*-12 CLA

## 1. Introduction

In the last decade, conjugated linoleic acid (CLA) has been studied intensively because of its unusual biological activities [1]. One of the most interesting aspects of CLA is its ability to reduce body fat while increasing lean body mass [2]. As part of the mechanism for these activities, it has been suggested that CLA reduces heparin-releasable lipoprotein lipase (HR-LPL) activity in adipocytes, and in fact this has been demonstrated in 3T3-L1 adipocyte cell culture model [2]. CLA also enhances fatty acid  $\beta$ -oxidation in muscle, which indicates increased use of fat as an energy source [2]. Since CLA is a mixture of isomers, it is of interest to determine which isomer is responsible for these biological activities. Previously we showed that

the *trans*-10,*cis*-12 isomer of CLA (one of two major isomers present in synthetically prepared CLA) is responsible for its reduction in body fat and for inhibition of HR-LPL activity in 3T3-L1 adipocytes [3]. This isomer is also responsible for the inhibition of stearoyl-CoA desaturase (SCD) [4], and reducing apolipoprotein B (apo B) secretion in cultured HepG2 cells [5]. Thus it is of interest whether this CLA isomer may also be solely responsible for inhibition of HR-LPL activity. To determine the key structural feature of *trans*-10,*cis*-12 CLA, we tested fatty acids that are structurally related to *trans*-10,*cis*-12 CLA using 3T3-L1 adipocytes. We also tested these compounds on glycerol release and intracellular triacylglyceride (TG) to estimate lipolysis and total fat deposit, respectively. In addition, since previous findings indicate that a *trans*-10 double bond is a key structure for inhibiting apo B secretion in HepG2 cells [5], we tested *trans*-10 18:1 on body compositional changes compared to *trans*-10,*cis*-12 CLA.

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## 2. Methods and materials

### 2.1. Materials

Triolein, [9,10-<sup>3</sup>H (N)] triolein (specific activity 12 Ci/μmol) was obtained from American Radioabeled Chemicals Incorporation (St. Louis, MO) and [1-<sup>14</sup>C] linoleic acid (specific activity 55 mCi/μmol) was obtained from Amer-sham Life Science (Arlington Heights, IL). 3T3-L1 preadipocytes were purchased from American Type Culture Collection (Rockville, MD). *trans-9,cis-12* octadecadienoic acid (31.7%), *cis-12* octadecenoic acid (98.1%), *trans-10* octadecenoic acid (89.9% for cell culture and 85.1% for animal feeding study), 11-(2'-(*n*-pentyl)phenyl)-10-undecylenic acid (P-110, 95%, E:Z about 7:3), *trans-10,cis-12/cis-9,trans-11*-octadecadien-1-ol (CLA-Alc., 95%), *trans-10,cis-12/cis-9,trans-11*-octadecadienamide (CLA-amide, 95%), 1-chloro-*trans-10,cis-12*-octadecadiene (CLA-Cl, 95%), and *cis-12*-octadecen-10-ynoic acid (10y,*cis-12*, 94.5%) were prepared by chemical synthesis by Dr. Sih and colleagues, at the Department of Pharmacy, University of Wisconsin–Madison. CLA was prepared as described [6]; the composition of CLA was 45.7% *cis-9,trans-11*, 47.6% *trans-10,cis-12*, 1.71% *trans,trans*, 3.04% other isomer. In addition, *cis-9,trans-11* (96.3%, 2.6% *trans,trans* isomer, and 1.08% others) and *trans-9,trans-11* (100%) CLA were purchased from Matreya Inc. (Pleasant Gap, PA). Natural Lipids (Hovdebygd, Norway) kindly provided *trans-10,cis-12* CLA (92.8% *trans-10,cis-12*, 1.61% *cis-9,trans-11*, 1.16% *trans,trans*, and 1.64% others). We purchased *cis-10* (99%), and *trans-10* (99%) heptadecenoic acid and *cis-10* (99%), and *trans-10* (99%) nonadecenoic acid from Nu-Chek Prep Corporation (Elysian, MN), and purchased *trans-9* (100%), *trans-11* (100%), *trans-12* (100%), *trans-13* (100%), *cis-9* (99%), *cis-11* (100%) and *cis-13* (100%) octadecenoic acids from Sigma Chemical Co. (St. Louis, MO). Purity was checked by gas chromatography using a Hewlett-Packard 5890 series II chromatograph (Hewlett-Packard, Andover, MA) fitted with a flame ionization detector and 3396A integrator. A Supelcowax-10 fused silica capillary column (60 m × 0.32 mm, inner diameter film thickness 0.25 μm) was used and oven temperature was programmed from 50° to 190°C, increased 20°C/min, held for 40 minutes, increased 10°C/min to 220°C, and held for 20 minutes.

### 2.2. Cell culture

The 3T3-L1 preadipocytes were cultured as described [7]. Briefly, 3T3-L1 preadipocytes were grown to confluence at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). At 2 days postconfluence (designated day 0), cell differentiation was induced with a mixture of methylisobutylxanthin (0.5 mmol/L), dexamethasone (0.25 μmol/L), and insulin (1 μg/mL) in DMEM containing 10% FBS. On day 2 this medium was replaced with

medium containing 10% FBS and insulin only. On day 4 and thereafter the medium consisted of DMEM plus 10% FBS only; this medium was subsequently replaced with fresh medium at 2-day intervals. Fatty acid–albumin complexes were prepared as described [2] and added to culture media for 48 hours at day 6, and cells were harvested at day 8. Final concentrations of fatty acids are indicated in each figure legend. All dishes including control had a final concentration of 100 μmol/L albumin. For the experiments with CLA derivatives, test compounds as well as CLA were dissolved in ethanol. In these experiments, control and treatment dishes all contained the same concentration of albumin (with no fatty acid) and ethanol (final concentration of 0.3%). At harvest, medium was collected, centrifuged to remove cell debris, and used for glycerol release analysis. Cells were then washed three times with phosphate buffered saline (PBS) and incubated with heparin containing DMEM (10 U/mL) for 1 hour, and the media was used for determining IIR-LPL activity. Subsequently, cells were washed three times with PBS, scraped, and sonicated to give homogenous samples for TG determination. LPL activity (EC 3.1.1.34) was measured as described [8]. Briefly, concentrated substrate was prepared by combining 300 mg triolein, 18 mg lecithin, and 200 μL of [<sup>3</sup>H] triolein in 5 mL glycerol and homogenizing. Fresh assay substrate was prepared by mixing 1 part concentrated substrate, 4 parts 0.2 mol/L Tris/HCl (pH 8.0) with 3% bovine serum albumin, and 1 part heat inactivated fasted rat serum. The reaction was started by adding 100 μL of samples to the same volume of assay substrate. After incubating at 37°C for 15 minutes, the assay was stopped by adding 3.25 mL methanol:chloroform:heptane solution (1.41:1.25:1). Subsequently, 1.05 mL 0.1 mol/L potassium carbonate/borate buffer (pH 10.5) was added, and the tubes were shaken vigorously and centrifuged at 3000 rpm for 20 minutes. A 200-μL quantity of the upper layer was used for radioactivity count. Nonreacted assay substrate was also used to get total count from each experiment. Recovery of free fatty acid was estimated at 71% using [<sup>14</sup>C] linoleic acid.

Free and esterified glycerol were determined in the medium and cell sonicates using a Sigma Diagnostic Kit (GPO Trinder, St. Louis, MO). This kit used a two-step process; free glycerol was measured without lipase, then lipase was added to hydrolyse esterified glycerol. The difference between the amounts of free and total glycerol is esterified glycerol. Protein was determined in LPL and TG samples using Bio-Rad DC Protein assay kit (Hercules, CA).

### 2.3. Animal studies and body composition analyses

Male retired breeder ICR mice and semipurified diet (TD94060, 99% basal mix) were purchased from Harlan Sprague-Dawley and Harlan Teklad (Madison, WI), respectively. The diet was composed as follows (ingredient, g/kg): sucrose, 476; casein, vitamin-free test, 210; corn starch, 150; DL-methionine, 3; corn oil 55; cellulose, 50; mineral mix, AIN-76, 35; vitamin mix, AIN-76A, 13; calcium car-

Table 1  
Effects of *trans*-10 octadecenoic acid on body composition in mice

	ECW (g)	% Fat	% Water	% Protein	% Ash
Control	38.2 ± 1.3	12.71 <sup>a</sup> ± 1.69	59.3 <sup>ab</sup> ± 1.2	20.83 <sup>ab</sup> ± 0.62	3.70 ± 0.06
t10:c12 CLA	37.8 ± 1.9	7.67 <sup>b</sup> ± 1.11	63.0 <sup>a</sup> ± 0.8	21.77 <sup>a</sup> ± 0.27	3.88 ± 0.16
t10-18:1	40.4 ± 1.2	15.33 <sup>a</sup> ± 1.50	57.9 <sup>b</sup> ± 1.3	19.74 <sup>b</sup> ± 0.41	3.88 ± 0.19

Twenty male retired breeder ICR mice were used. After a 5-day adaptation period mice were randomly separated into three groups and fed control diet, diet supplemented with 0.27% *trans*-10, *cis*-12 CLA (t10:c12 CLA), or diet supplemented with 0.27–0.29% *trans*-10 octadecenoic acid (t10-18:1) for 3 weeks. Numbers are mean ± SE (n = 5). Means with different superscript letters in each column are significantly different at  $P < 0.05$ . CLA = conjugated linoleic acid; ECW = empty carcass weight.

bonate, 4; choline bitartrate, 2; and ethoxyquin, 0.1. The mineral mix (AIN-76) was composed as follows (ingredient, g/kg): calcium phosphate (dibasic), 500; potassium citrate (monohydrate), 220; sodium chloride, 74; potassium sulfate, 52; magnesium oxide, 24; ferric citrate, 6.0; manganese carbonate, 3.5; zinc carbonate, 1.6; cupric carbonate, 0.3; chromium potassium sulfate, 0.55; potassium iodate, 0.01; sodium selenite, 0.01; and sucrose, 118. The vitamin mix (AIN-76A) was composed as follows (ingredient, g/kg): thiamin HCl, 0.6; riboflavin, 0.6; pyridoxine HCl, 0.7; niacin, 3.0; calcium pantothenate, 1.6; folic acid, 0.2; biotin, 0.02; vitamin B<sub>12</sub> (0.1% trituration in mannitol), 1.0; dry vitamin A palmitate (500,000 U/g), 0.8; dry vitamin E acetate (500 U/g), 10.0; vitamin D<sub>3</sub>, trituration (400,000 U/g), 0.25; menadione sodium bisulfite complex, 0.15, and sucrose, 981.08.

Supplemental CLA (5 g/kg) was added to diets at the expense of corn oil. Diet was stored at  $-20^{\circ}\text{C}$  until use. Mice were housed individually in wire-bottomed cages in a windowless room with a 12-h light-dark cycle in strict accordance with guidelines established by the Research Animal Resources Center of University of Wisconsin–Madison. Diet and water, available ad libitum, were freshly provided three times per week. After a 5-day adaptation period, mice were randomly separated into groups and fed control diet, diet supplemented with *trans*-10, *cis*-12 CLA (0.27%), or diet supplemented with *trans*-10 octadecenoic acid (0.29%) for 3 weeks.

For body composition analyses, animals were sacrificed, gut contents were removed (to obtain empty carcass weight [ECW]), and the carcasses frozen at  $-20^{\circ}\text{C}$ . Frozen carcasses were chopped and freeze-dried to determine water content. Each dried carcass was ground to give a homogeneous sample before further analysis. Total nitrogen was analyzed by the Kjeldahl method [9] by the Department of Soil Science, University of Wisconsin–Madison. Carcass fat content was measured by extraction with diethyl ether overnight using a Soxhlet apparatus. Total ash content was determined by incineration (500–600°C, overnight).

## 2.4. Statistical analyses

One-way analysis of variance was performed on data presented in Table 1, and two-way analysis of variance

(treatments and experiments) was performed on data depicted in the figures (individually cited later here). Of major interest here are the comparisons among the treatments; these were computed using the Statistical Analysis System (SAS Institute Inc., Cary, NC) with the general linear mean procedure and least square means options. If the interaction between treatment and experiment was significant, this interaction was then used as the error term in the least square means analysis.

## 3. Results

The concentrations used in this report are based on the finding that sera CLA levels of rats fed diet supplemented with 0.5% CLA for 28 days were 72  $\mu\text{mol/L}$  (range 23–120  $\mu\text{mol/L}$ ) [2]. Thus in this study, we used 50  $\mu\text{mol/L}$  for single isomers and 100  $\mu\text{mol/L}$  for mixed isomers.

Experiments were done in multiple sets of quadruplicates. Figures 1A–1D show inhibition of HR-LPL activity by various 18-carbon fatty acids. As previously reported, the *trans*-10, *cis*-12 isomer of CLA consistently and significantly reduced HR-LPL activity in 3T3-L1 adipocytes [3]. This effect is shown in all four sets of experiments and used as a negative control. In contrast the monounsaturated octadecenoic acids (18:1) with double bonds at *cis*-9, *cis*-13, *trans*-12, *trans*-13 (Fig. 1A), *cis*-11, *trans*-11, *trans*-9 (Fig. 1B), *cis*-12 (Fig. 1C), or *trans*-10 (Fig. 1D) exhibited no effects on HR-LPL activity. In addition the linoleic acid isomer *trans*-9, *cis*-12 18:2 (Fig. 1D), 10y, *cis*-12 (an 18-carbon structure similar to *trans*-10, *cis*-12 CLA but with a triple bond instead of a double bond at C:10) (Fig. 1C), and P-10 (11-(2-(*n*-pentyl)phenyl)-10-undecylenic acid) (a *trans*-10, *cis*-12 CLA related structure in which the *trans*-10 double bond is part of a benzene ring) did not exhibit any effect on HR-LPL activity (Fig. 1B). The effect of *cis*-9 18:1 (oleic acid) was marginal in that it enhanced HR-LPL activity in three experiments but induced no significant effect in three comparable experiments.

To determine the effects of these CLA cognates on lipolysis and fat deposition, respectively, we measured glycerol release into media and the amount of TG within cells. As previously reported [3], *trans*-10, *cis*-12 CLA increased lipolysis in this model. The 18:1 compounds with double



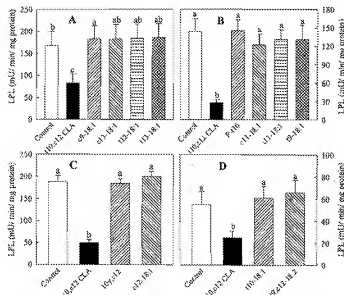


Fig. 1. Effects of 18-carbon fatty acids on heparin-releasable lipoprotein lipase (HR-LPL) activity in 3T3-L1 adipocytes. At day 6 of differentiation, cells were treated with these compounds as an albumin complex for 48 hours. All dishes including control contained the same concentration of albumin (100  $\mu\text{mol/L}$ ). Numbers are mean  $\pm$  SE,  $n = 23$ –24 for (A) from six independent experiments,  $n = 10$ –12 for (B) and (C) from three independent experiments, and  $n = 7$ –8 for (D) from two independent experiments. Means with different letters in each figure are significantly different at  $P < 0.05$ . Abbreviations and final concentrations used are as follows: (A)  $\Delta^{10}$ ,  $\Delta^{12}$  CLA, *trans*-10,*cis*-12 conjugated linoleic acid, 46  $\mu\text{mol/L}$ ; *cis*-9:18:1, *cis*-9 octadecenoic acid, 50  $\mu\text{mol/L}$ ;  $\Delta^{13}$ :18:1, *cis*-13 octadecenoic acid, 50  $\mu\text{mol/L}$ ;  $\Delta^{12}$ :18:1, *trans*-12 octadecenoic acid, 50  $\mu\text{mol/L}$ ;  $\Delta^{13}$ :18:1, *trans*-13 octadecenoic acid, 50  $\mu\text{mol/L}$ ; (B) P-10, 11-(2-(4-pentylphenyl)-10-undecylenic acid, 48  $\mu\text{mol/L}$ ;  $\Delta^{11}$ :18:1, *cis*-11 octadecenoic acid, 50  $\mu\text{mol/L}$ ;  $\Delta^{11}$ :18:1, *trans*-11 octadecenoic acid, 50  $\mu\text{mol/L}$ ;  $\Delta^{12}$ :18:1, *trans*-12 octadecenoic acid, 50  $\mu\text{mol/L}$ ; (C)  $\Delta^{10}$ ,  $\Delta^{12}$ , *cis*-10 octadecenoic acid, 47  $\mu\text{mol/L}$ ;  $\Delta^{12}$ :18:1, *cis*-12 octadecenoic acid, 49  $\mu\text{mol/L}$ ; and (D)  $\Delta^{10}$ :18:1, *trans*-10 octadecenoic acid, 45  $\mu\text{mol/L}$ ;  $\Delta^{9}$ ,  $\Delta^{12}$ :18:2, *trans*-9,*cis*-12 octadecadienoic acid, 32  $\mu\text{mol/L}$ .

bonds at the *trans*-12 (Fig. 2A), *cis*-11, *trans*-11, or *trans*-9 (Fig. 2B) had no effect on glycerol release. However, the 18:1 compounds with double bonds at *cis*-9 (Fig. 2A) reduced glycerol release, whereas 18:1 with double bonds at *cis*-13, *trans*-13 (Fig. 2A) (Fig. 2C) or *trans*-10 positions (Fig. 2D) enhanced glycerol release. *Trans*-9, *cis*-12 18:2 had no effect on glycerol release (Fig. 2D). P-10 increased (Fig. 2B) and 10y, *cis*-12 (Fig. 2C) decreased glycerol release in this system. Hence the structural feature or features involved in regulating lipolysis appear to be complex.

Total intracellular TG was reduced by *trans*-10,*cis*-12 CLA as reported previously [3]. All 18-carbon monounsaturated fatty acids tested had no effect on total TG (Fig. 3A–3D). No effect on TG was observed by treating with 10y, *cis*-12 (Fig. 3C), P-10 (Fig. 3B), or *trans*-9, *cis*-12 18:2 (Fig. 3D).

We also tested CLA derivatives that have functional groups other than carboxylic acid. Compounds with alcohol, amide, and chloride groups were prepared and were tested for effects on HR-LPL activity, glycerol release, and intra-

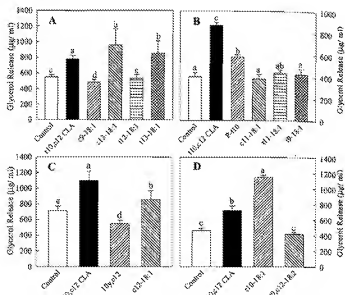


Fig. 2. Effects of 18-carbon fatty acids on glycerol release in 3T3-L1 adipocytes. At day 6 of differentiation, cells were treated with these compounds as an albumin complex for 48 hours. At the end of incubation, culture medium was collected and analyzed for free glycerol after centrifugation. All dishes including control contained the same concentration of albumin (100  $\mu\text{mol/L}$ ). Numbers are mean  $\pm$  SE,  $n = 23$ –24 for (A) from six independent experiments,  $n = 10$ –12 for (B) and (C) from three independent experiments, and  $n = 7$ –8 for (D) from two independent experiments. Means with different letters in each figure are significantly different at  $P < 0.05$ . For abbreviations and final concentrations used, see legend to Fig. 1.

cellular TG. Since these compounds do not have the carboxylic group needed to complex with albumin, they were dissolved in ethanol before addition to the culture medium. The HR-LPL activity in cells treated with CLA dissolved in ethanol was not significantly different than when CLA was treated complexed to albumin (Fig. 4B). The alcohol form of CLA slightly reduced HR-LPL activity while the amide form of CLA (Fig. 4A), as well as the chloride form of CLA (Fig. 4B) had no effect on HR-LPL activity.

Glycerol release was increased by CLA and reduced by the alcohol and amide forms of CLA (Fig. 5A). The chloride form of CLA did not have any effect on glycerol release (Fig. 5B). Total TG was reduced by *trans*-10,*cis*-12 CLA as well as the alcohol form of CLA, but not by the amide and chloride forms of CLA (Figs. 6A and 6B).

Of the 17- and 19-carbon monounsaturated fatty acids with a double bond at the 10th position (either *cis* or *trans* configuration) that were studied, only *cis*-10 17:1 inhibited HR-LPL activity, but it was less active than *trans*-10,*cis*-12 CLA (Fig. 7A). In addition, *cis*-10 17:1 increased glycerol release whereas *trans*-10 17:1, *cis*-10 19:1 or *trans*-10 19:1 decreased glycerol release (Fig. 7B). *Cis*-10 17:1 and *cis*-10 19:1 slightly reduced cellular TG compared to control, but *trans*-10 17:1 and *trans*-10 19:1 were without significant effect (Fig. 7C).

Since *trans*-10 18:1 exhibited an effect on glycerol release, we also tested whether *trans*-10 octadecenoic acid

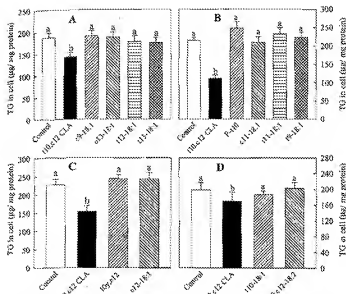


Fig. 3. Effects of 18-carbon fatty acids on esterified glycerol in 3T3-L1 adipocytes. At day 6 of differentiation, cells were treated with these compounds as an albumin complex for 48 hours. All dishes including control contained the same concentration of albumin (100 µmol/L). Cells were scraped and sonicated to determine triacylglyceride (as esterified glycerol) and protein. Numbers are mean  $\pm$  SE,  $n = 23$ –24 for (A) from 6 independent experiments,  $n = 10$ –12 for (B) and (C) from three independent experiments, and  $n = 7$ –8 for (D) from two independent experiments. Means with different letters in each figure are significantly different at  $P < 0.05$ . For abbreviations and final concentrations used, see legend to Fig. 1.

might induce body compositional changes similar to those induced by the *trans*-10,*cis*-12 CLA isomer (Table 1). At the end of the experimental period the group fed diet sup-

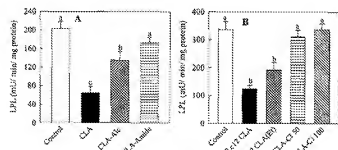


Fig. 4. Effects of CLA derivatives on heparin releasable lipoprotein lipase (HL-LPL) activity in 3T3-L1 adipocytes. At day 6 of differentiation, cells were treated with these compounds for 48 hours. Except for t10,c12-CLA, all treatment compounds were dissolved in ethanol. All dishes including t10,c12-CLA and control contained the same concentration of albumin (100 µmol/L) and ethanol (0.3% final concentration). Numbers are mean  $\pm$  SE,  $n = 6$ –8 from two independent experiments. Means with different letters in each figure are significantly different at  $P < 0.05$ . Abbreviations and final concentrations used are as follows: (A) CLA, *trans*-10,*cis*-12 (48%) and *cis*-9,*trans*-11 (46%) isomers, 98 µmol/L; CLA-Alc., *trans*-10,*cis*-12/*cis*-9,*trans*-11-octadecadienyl-1-ol, 95 µmol/L; CLA-amide, *trans*-10,*cis*-12/*cis*-9,*trans*-11-octadecadienamide, 95 µmol/L, and (B) t10,c12-CLA, *trans*-10,*cis*-12 conjugated linoleic acid, 46 µmol/L. Et indicates dissolved in ethanol before treating to cells; CLA-Cl, 1-chloro-*trans*-10,*cis*-12-octadecadiene, 48 or 96 µmol/L.

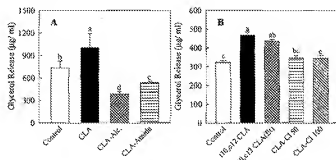


Fig. 5. Effects of CLA derivatives on glycerol release in 3T3-L1 adipocytes. At day 6 of differentiation, cells were treated with these compounds as an albumin complex for 48 hours. Except for t10,c12-CLA, all treatment compounds were dissolved in ethanol. All dishes including t10,c12-CLA and control contained the same concentration of albumin (100 µmol/L) and ethanol (0.3% final concentration). At the end of incubation culture medium was collected and analyzed for free glycerol after centrifugation. Numbers are mean  $\pm$  SE,  $n = 6$ –8 from two independent experiments. Means with different letters in each figure are significantly different at  $P < 0.05$ . For abbreviations and final concentrations used, see legend to Fig. 4.

plemented with *trans*-10,*cis*-12 CLA exhibited significantly reduced body fat (40% reduction compared to control), whereas whole body water was significantly enhanced and whole body protein, and ash were apparently enhanced. By contrast, the group fed *trans*-10 octadecenoic acid exhibited no significant change in any of these measurements (Table 1). There were no significant difference in mean body weights (control group,  $40.5 \pm 1.2$  g; *trans*-10,*cis*-12 CLA-fed group,  $40.3 \pm 2.2$  g; and t10 18:1-fed group,  $42.6 \pm 1.3$  g) or mean total feed intake (control group,  $91.2 \pm 4.1$  g; *trans*-10,*cis*-12 CLA-fed group,  $83.7 \pm 2.4$  g; and t10 18:1-fed group,  $89.8 \pm 4.2$  g).

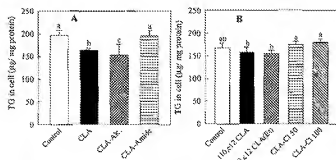


Fig. 6. Effects of CLA derivatives on esterified glycerol in 3T3-L1 adipocytes. At day 6 of differentiation, cells were treated with these compounds for 48 hours. Except for t10,c12-CLA, all treatment compounds were dissolved in ethanol. All dishes including t10,c12-CLA and control contained the same concentration of albumin (100 µmol/L) and ethanol (0.3% final concentration). Cells were scraped and sonicated to determine triacylglyceride (as esterified glycerol) and protein. Numbers are mean  $\pm$  SE,  $n = 6$ –8 from two independent experiments. Means with different letters in each figure are significantly different at  $P < 0.05$ . For abbreviations and final concentrations used, see legend to Fig. 4.

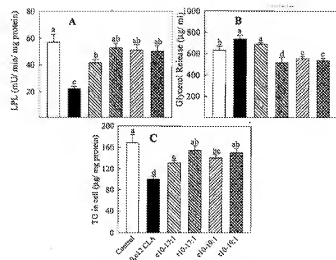


Fig. 7. Effects of odd-numbered fatty acids on heparin-releasable lipoprotein lipase (HR-LPL) activity (A), glycerol release (B), and cellular esterified glycerol (C) in 3T3-L1 adipocytes. At day 6 of differentiation, cells were treated with 50  $\mu\text{mol/L}$  of fatty acid for 48 hours. All treatments had 100  $\mu\text{mol/L}$  of albumin. Reported values are mean  $\pm$  SE ( $n = 12$ , collected from three independent experiments). Means with different letters in each figure are significantly different at  $P < 0.05$ . CLA, conjugated linoleic acid; c, *cis*; t, *trans*; 17:1, heptadecenoic acid; 19:1, nonadecenoic acid.

#### 4. Discussion

Table 2 summarizes the results of this study along with those of previously published investigations [2,3,10–12]. Among octadecadienoic acids only *trans*-10,*cis*-12 CLA reduced the activity of HR-LPL, whereas *cis*-9,*trans*-11, *trans*-9,*trans*-11, linoleic acid, or *trans*-9,*cis*-12 octadecadienoic acid did not reduce HR-LPL activity. Various 18-carbon monounsaturated fatty acids with double bonds located between carbons 9 and 13, in either *trans* or *cis* configurations, did not inhibit HR-LPL activity. CLA amide and chloride derivatives were without effect, whereas CLA alcohol exhibited a modest inhibitory effect that was less than that seen with *trans*-10,*cis*-12 CLA. We propose that this is due to the conversion of the alcohol form to free acid form; hence the effect of the alcohol form of CLA is less active than CLA itself. The data support the conclusion that the *trans*-10,*cis*-12 conjugated double bond in conjunction with a carboxyl group at C-1 is required for inhibition of HR-LPL activity.

The TG content in fat cells reflects the balance between fat uptake by HR-LPL, lipogenesis, and lipolysis. Our data consistently show that any compound that reduced HR-LPL activity in adipocytes also reduced body fat in animals. Since the amount of protein secreted into heparin containing medium was not different between treatment groups, it is unlikely that the effect of CLA on HR-LPL inhibition results from less protein secretion. Therefore the effects of CLA on HR-LPL can be due to regulation of HR-LPL expression, modification of location of HR-LPL, or interaction with HR-LPL directly.

Unlike its consistent inhibition of HR-LPL activity, the

effects of CLA on intracellular LPL activity vary, either inhibiting [13] or having no effect [12]. We were able to observe inhibitory effects of CLA (as a mixture of *trans*-10,*cis*-12 and *cis*-9,*trans*-11 isomers) on HR-LPL activity in as short a time as 20 minutes (unpublished observations, [14]). This appears to be too rapid for a transcriptional effect; the effect of CLA on HR-LPL activity may result in part from direct interaction between *trans*-10,*cis*-12 CLA and LPL, similar to the inhibition mechanism of CLA on stearoyl CoA desaturase (SCD,  $\Delta$ -9 desaturase) [4]. Alternatively, CLA might inhibit LPL enzyme relocation to membrane, making less LPL available for the activity. In addition, we cannot rule out the possibility that CLA may further interact at the transcriptional level, which has been reported by others, for example reduced LPL mRNA in 3T3-L1 adipocytes or adipose tissues by *trans*-10,*cis*-12 CLA [14–16].

Previously, Lin et al. [12] reported that both *trans*-10,*cis*-12 and *cis*-9,*trans*-11 CLA isomers reduced HR-LPL activity from 3T3-L1 adipocytes, although the concentrations were much higher than those we used in this study. When similar concentrations were compared there was no effect by *cis*-9,*trans*-11 CLA, whereas *trans*-10,*cis*-12 CLA effectively reduced HR-LPL activity.

CLA may also affect lipogenesis in adipocytes. Previously our group reported that CLA did not affect activities of acetyl-CoA carboxylase and fatty acid synthetase (key enzymes involved in lipogenesis), both in vivo and during 1 week of feeding [13] and in 3T3-L1 adipocytes [17]. This is consistent with observations by Bee [18]. However, others report that CLA reduced activities and/or mRNA level of these two enzymes in adipocytes [15,16,19–21]. Thus CLA may result in less TG in adipocytes by reducing lipogenesis; however, we did not measure lipogenesis in these experiments.

Glycerol release represents lipolysis in adipocytes. Our results here indicate that glycerol release did not correlate well with TG content in cells or with reduced body fat in vivo. This may be due to the sensitivity of the method used in our experiments. However, nordihydroguaiaretic acid (NDGA) and AA861, both well known inhibitors of lipoxigenase and hormone-sensitive lipase, decreased lipolysis in our experiments, as expected, compared to control (as  $\mu\text{g}$  glycerol/mL: control  $181 \pm 8$ , NDGA ( $1 \times 10^{-5}$  mol/L)  $131 \pm 9$ , AA861 ( $1 \times 10^{-6}$  mol/L)  $150 \pm 22$ , and CLA  $237 \pm 11$ ,  $n = 7-8$  from two independent experiments). This suggests that this method represents the effects of treatment in this model. This was accompanied by reduced TG (as  $\mu\text{g}/\text{mg}$  protein: control  $153 \pm 13$ , NDGA ( $1 \times 10^{-5}$  mol/L)  $93 \pm 10$ , AA861 ( $1 \times 10^{-6}$  mol/L)  $95 \pm 7$ , and CLA  $94 \pm 6$ ,  $n = 7-8$  from two independent experiments) and reduced body fat in mice for NDGA (for experimental conditions, see [22]). However, compounds that increased glycerol release only did not result in decreased TG, for example, *trans*-10 18:1 effectively increased glycerol release without affecting HR-LPL activity in 3T3-L1 adipocytes and had no

Table 2  
Summary of compounds used

Compound	Inhibition of HR-LPL	Glycerol release	Total TG
<b>Octadecadienoic acid</b>			
<i>trans</i> -10, <i>cis</i> -12 CLA	+++	↑	↓
<i>cis</i> -9, <i>trans</i> -11 CLA [3,12]	– [3]/++ [12]	↑	↓
<i>trans</i> -9, <i>trans</i> -11 CLA [3]	–	↑	↓
<i>cis</i> -9, <i>cis</i> -12 octadecadienoic acid (linoleic acid) [2,3]	–	↑	↑
<i>trans</i> -9, <i>cis</i> -12 octadecadienoic acid	–	–	–
<b>Octadecenoic acid</b>			
<i>cis</i> -9 octadecenoic acid (oleic acid)	–	↓	–
<i>cis</i> -11 octadecenoic acid	–	–	–
<i>cis</i> -12 octadecenoic acid	–	↑	–
<i>cis</i> -13 octadecenoic acid	–	↑	–
<i>trans</i> -9 octadecenoic acid	–	–	–
<i>trans</i> -10 octadecenoic acid	–	↑ ↑	–
<i>trans</i> -11 octadecenoic acid (vaccenic acid)	–	–	–
<i>trans</i> -12 octadecenoic acid	–	↑	–
<i>trans</i> -13 octadecenoic acid	–	↑	–
<b>Eicosadienoic acid</b>			
<i>Cis</i> -11, <i>cis</i> -14 eicosadienoic acid [10]	–	–	–
<i>Cis</i> -11, <i>trans</i> -13 and <i>trans</i> -12, <i>cis</i> -14 CEA [10]	++	↑	↓
<b>CLA derivatives</b>			
<i>trans</i> -10, <i>cis</i> -12/ <i>cis</i> -9, <i>trans</i> -11-octadecadien-1-ol (CLA-Alc.)	+	↓	↓
<i>trans</i> -10, <i>cis</i> -12/ <i>cis</i> -9, <i>trans</i> -11-octadecadienamide (CLA-Amide)	–	–	–
1-chloro- <i>trans</i> -10, <i>cis</i> -12-octadecadiene (CLA-Cl)	–	↓	–
<b>Odd-numbered fatty acids</b>			
<i>cis</i> -10 heptadecenoic acid	+	↑	↓
<i>trans</i> -10 heptadecenoic acid	–	↓	↓
<i>cis</i> -10 nonadecenoic acid	–	↓	↓
<i>trans</i> -10 nonadecenoic acid	–	↓	–
<i>Cis</i> -10, <i>cis</i> -13 nonadecadienoic acid [11]	–	↓	–
<i>Cis</i> -10, <i>trans</i> -12 and <i>trans</i> -11, <i>cis</i> -13 CNA [11]	++++	↑	↓
<b>Others</b>			
<i>cis</i> -12-octadecen-10-ynoic acid (10y, <i>cis</i> -12)	–	↓	–
11-(2-( <i>n</i> -pentyl)phenyl)-10-undecylenic acid ( <i>P</i> -110)	–	↑	–

– = effect; + = inhibition; ↑ = increase; ↓ = decrease.

No numbers in square brackets indicate references from which data are collected.

effect on total TG or body fat in mice. This suggests that this lipolysis assay method may not be sensitive enough to enable us to observe the difference in TG; thus further studies are need to clarify the correlation between lipolysis and TG in this cell culture model.

Previously we reported that the *trans*-10,*cis*-12 CLA isomer effectively inhibited hepatic SCD [4]. In addition, *trans*-10,*cis*-12 CLA is also responsible for decreased apo B secretion in HepG2 cells, although a *trans*-10 double bond is a key factor for this activity [5]. It was of interest that odd-numbered fatty acids such as CNA [11] can inhibit HR-LPL activity as well as reduce the TG in the cell. Since CNA and CLA cannot share common metabolites because of the difference of one carbon, we proposed that CLA itself was directly effective. In this study, we observed that *cis*-10 17:1 inhibits HR-LPL activity, although less so than *trans*-10,*cis*-12 CLA, indicating that a double bond at the 10th position may play an important role in these activities.

It appears, then, that *trans*-10,*cis*-12 CLA is directly responsible for reduction of HR-LPL activity, and that the

*trans*-10 double bond may be the key. In contrast, either a *trans*-10 or *cis*-12 bond may induce glycerol release from adipocytes. This study also suggests that lipolysis may not be a good indicator of predicting TG content in the 3T3-L1 adipocyte model; rather, inhibition of HR-LPL activity may provide a better prediction of TG content in this cell line and fat level in animal models.

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## Inhibition of hepatic stearoyl-CoA desaturase activity by *trans*-10,*cis*-12 conjugated linoleic acid and its derivatives

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### Abstract

Conjugated linoleic acid (CLA) has been reported to decrease stearoyl-CoA desaturase (SCD) activity by decreasing mRNA expression. This investigation was designed to determine whether structurally related compounds of CLA have a direct inhibitory effect on SCD activity. *Trans*-10,*cis*-12 CLA had strong inhibitory activity on SCD while *cis*-9,*trans*-11, and *trans*-9,*trans*-11 isomers had no effect. *Trans*-10 octadecenoate was not inhibitory, whereas *cis*-12 octadecenoate was inhibitory, but not as effective as *trans*-10,*cis*-12 CLA. Of the oxygenated derivatives, 9-peroxy-*cis*/*trans*-10, *trans*-12 octadecadienoate was a more effective inhibitor than *trans*-10,*cis*-12 CLA, whereas 9-hydroxy-*trans*-10, *cis*-12 octadecadienoate was less effective. Interestingly, *cis*-11 octadecadienoate and *cis*-12 octadecen-10-ynoate were slightly inhibitory. However, *trans*-9 and *trans*-11 octadecenoates, and *trans*-9,*cis*-12 octadecadienoate were all inactive under test condition, as were linoleate, oleate, and arachidonate. Derivatives of CLA acid modified to alcohol, amide or chloride were all inactive. A *cis*-12 double bond appears to be a key structural feature for inhibiting SCD activity, especially when coupled with a *trans*-10 double, whereas a *cis*-11 double bond is less effective. © 2000 Elsevier Science B.V. All rights reserved.

**Keywords:** Conjugated linoleic acid; Stearoyl-CoA desaturase;  $\Delta^9$  desaturase; *trans*-10,*cis*-12 CLA

### 1. Introduction

Abbreviations: SCD, stearoyl-CoA desaturase; CLA, conjugated linoleic acid; 9-HODE, 9-hydroxy-*trans*-10,*cis*-12 octadecadienoic acid; 9-HOO, 9-hydroperoxy *trans*-10,*cis*-12 octadecadienoic acid; CLA-Alc, *trans*-10,*cis*-12/*cis*-9,*trans*-11-octadecadien-1-ol; CLA-amide, *trans*-10,*cis*-12/*cis*-9,*trans*-11-octadecadienamide; CLA-Cl, 1-chloro-*trans*-10,*cis*-12-octadecadiene; 10y,*cis*-12, *cis*-12-octadecen-10-ynoic acid; AA, arachidonic acid; LA, linoleic acid; ACS, acyl CoA synthetase; LPL, lipoprotein lipase; PUFA, polyunsaturated fatty acid

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CLA refers to a group of geometric and positional isomers of conjugated linoleic acid. CLA was originally isolated from ground beef as an anticarcinogenic principal which contained mainly the *cis*-9,*trans*-11 isomer [1,2]. This isomer can be formed during the biohydrogenation of linoleic acid to stearic acid by rumen bacteria [3]. Alternatively, the latest research shows that it can form in animals via  $\Delta^9$  desaturation of *trans*-11 octadecenoic acid (*trans* vaccenic acid)

[4,5]. CLA typically used in animal studies is prepared by alkali isomerization from pure linoleic acid, and contains more than 95% CLA, mainly *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers (85–90%) along with other minor isomers (*trans*,*trans* or *cis*,*cis*). This preparation of CLA has shown a wide range of biologically beneficial activities: reduction of carcinogenesis in several animal models [1,6–8]; decreased severity of atherosclerosis [9,10]; reduction of adverse effects of immune stimulation [11,12]; growth promotion in young rats [13]; and a reduction of body fat and an increase in lean body mass in several animal species [14–18]. However, depending on the source of linoleic acid (pure fatty acid or vegetable oil) and conditions of isomerization, the isomer distribution can vary significantly [19,20]. In some studies, CLA preparations contained a more complex mixture of isomers, such as 8,10 and 11,13 isomers as well as 9,11 and 10,12 isomers [19,20].

Since Ha et al. [6] reported only the *cis*-9,*trans*-11 isomer was incorporated in the phospholipid fraction of mouse forestomach, it has been postulated that *cis*-9,*trans*-11 CLA is the biologically active isomer. More recently, several investigators [17,21,22] reported that all CLA isomers are incorporated in both phospholipids and neutral lipids. However, it was not until 1998 that Lee et al. [23] reported the possible biological activity of the *trans*-10,*cis*-12 isomer on stearoyl-CoA desaturase (SCD). In the first part of that study, they fed CLA (as a mixture of 42% *cis*-9,*trans*-11 and 44% *trans*-10,*cis*-12) to mice and analyzed hepatic SCD mRNA expression, which was significantly decreased by CLA feeding. In the second part of the experiment, H2.35 mouse hepatocytes were tested. CLA generated by rumen bacteria (this preparation contained mainly *cis*-9,*trans*-11 CLA, >60% of total fatty acid, with no *trans*-10,*cis*-12 isomer) did not have any effect on SCD mRNA expression. Based on these observations, Lee et al. [23] suggested that the *trans*-10,*cis*-12 isomer was responsible for the effects of synthetic CLA on SCD. Recently, we clearly demonstrated that the *trans*-10,*cis*-12 isomer was responsible for body composition changes in mice and the decrease in lipoprotein lipase activity in 3T3-L1 adipocytes, while *cis*-9,*trans*-11 and *trans*-9,*trans*-11 isomers did not have any impact [24]. In the current study, we tested the direct effects of CLA on the activity of SCD using

high purity isomers. Other 18-carbon fatty acids, CLA derivatives, as well as arachidonic acid (a known inhibitor of SCD gene) were also compared with isomers of CLA.

## 2. Materials and methods

[1-<sup>14</sup>C]Palmitic acid (50 mCi/mmol) was purchased from American Radiolabeled Chemicals, (St. Louis, MO). 9-hydroxy-(*E,Z*)-(±)-10,12-octadecadienoic acid (9-HODE) was purchased from Cayman (Ann Arbor, MI). 9-Hydroperoxy-10,12 octadecadienoic acid (9-HOO), a mixture of *trans*-10,*cis*-12 and *trans*-10,*trans*-12 in 2:1, 95%), *trans*-9,*cis*-12 octadecadienoic acid (31.7%), *cis*-12 octadecadienoic acid (98.1%), *trans*-10 octadecadienoic acid (89.9%), *trans*-10,*cis*-12/*cis*-9,*trans*-11 octadecadien-1-ol (CLA-Alc, 95%), *trans*-10,*cis*-12/*cis*-9,*trans*-11 octadecadienamide (CLA-amide, 95%), 1-chloro-*trans*-10,*cis*-12 octadecadiene (CLA-Cl, 95%), and *cis*-12-octadecen-10-ynoic acid (10y,*cis*-12, 94.5%) were prepared by chemical synthesis. CLA was prepared as described [2]; the composition of CLA was 45.7% *cis*-9,*trans*-11, 47.6% *trans*-10,*cis*-12, 1.71% *trans*,*trans*, 3.04% other isomer. *Cis*-9,*trans*-11 CLA was purchased from Matreya (Pleasant Gap, PA); 96.3% *cis*-9,*trans*-11/*trans*-9,*cis*-11, 2.6% *trans*,*trans* isomer, and 1.08% others. Natural Lipids (Hovdebygd, Norway) kindly provided *trans*-10,*cis*-12 CLA; 92.8% *trans*-10,*cis*-12, 1.61% *cis*-9,*trans*-11, 1.16% *trans*,*trans*, and 1.64% others. Other fatty acids were purchased from Nu-Chek-Prep, (Elysian, MN). Weanling male ICR mice were purchased from Harlan Sprague Dawley (Madison, WI). Semi-purified diet was purchased from Harlan Teklad (TD96040, Madison, WI). Fat-free carbohydrate diet was from USB (fat-free diet, 15750 PT).

### 2.1. Preparation of liver microsomal fraction

Mice were housed in a windowless room with a 12-h light-dark cycle in strict accordance with guidelines established by the Research Animal Resources Center of University of Wisconsin-Madison and a protocol approved by the Animal Care Committee. Diet and water were available ad libitum. Animals were fed according to Miller and Ntambi [25]. Mice

were starved for 24 h, refed for 24 h, starved for 24 h, and then fed fat-free carbohydrate diet for 24 h before CO<sub>2</sub> suffocation. Microsomal fractions were prepared from mice livers as described previously [26] with little modification. Liver homogenate was prepared with 4 vols. (w/v) of 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4) and centrifuged at 12 000 × g for 15 min. The supernates were re-centrifuged at 100 000 × g for 1 h, pellets were rinsed once and resuspended before use (microsomal fractions). All preparations were made at 4°C. Three independent preparations of microsomal fractions were used. Protein was determined using Bio-Rad DC Protein assay kit.

The SCD activity was determined as described [27]. The assay system contained 7.2 mM ATP, 0.54 mM CoA, 6 mM MgCl<sub>2</sub>, 0.8 mM NADH, 0.1 M potassium phosphate buffer (pH 7.16), 204 nmol [1-<sup>14</sup>C]palmitic acid (specific activity 0.98 Ci/mol) in 20 µl ethanol, testing fatty acid complexed with albumin (final concentration of 0–100 µM, as indicated in figure legends) and microsomal fraction (0.5–1.8 mg protein) in 1 ml final volume. The reaction was initiated by the addition of microsomal fraction and incubated at 37°C for 15 min. All fatty acids were dissolved in KOH to complex with albumin before addition to the incubation mixture. 9-HODE, 9-HOO, and CLA derivatives (Fig. 4) were in ethanol solution as indicated in legends. For comparison, CLA was also dissolved in ethanol and tested. For the experiment with acyl CoA synthetase (ACS), ACS (8 mU) was pre-incubated for 5 min with cofactors and fatty acids and then incubated for 5 min with the microsomal fractions. The reaction was stopped by adding 1 ml 12% KOH in ethanol and heating for 45 min at 80°C. Fatty acids were extracted with hexane after acidification with HCl. Fatty acid methyl esters were prepared with 4% HCl/MeOH at 60°C for 20 min, and separated by thin-layer chromatography on silver nitrate-impregnated silica gel G plates [28] using a mixture of hexane:diethyl ether (9:1). To help visualization, cold standards (palmitic and palmitoleic acid methyl esters) were co-spotted with samples. After visualization with 2',7'-dichlorofluorescein, corresponding spots were scraped off the plates, extracted with hexane and subjected to liquid scintillation counting. Enzyme activities were calculated as nmol palmitic

acid converted to palmitoleic acid per min per mg protein.

## 2.2. Statistics

Because of the experimental structure, the data were divided into six groups so that data were balanced (or close to balanced) with each group. A balanced design means that within a group, the same treatments were used in all experiments in the group. Data were subjected to analysis using the Statistics Analysis System (SAS Users Guide: Statistics, SAS Institute, Cary, NC). Within each group, data were analyzed with PROC MIXED as a two-way analysis of variance with treatment as a fixed factor and experiment and the experiment by treatment interacts as random factors. The error term for comparing treatments was a mix of the residual error and the experiment by treatment interact as determined by PROC MIXED using the Satterthwaith procedure for degrees of freedom [29].

## 3. Results

Fig. 1 shows the effects of different CLA isomers and linoleic acid derivatives on SCD activity. CLA mixture at 100 µM (either complexed with albumin or dissolved in ethanol) significantly reduced the activity of SCD. Two major isomers of CLA, *cis*-9,*trans*-11 and *trans*-10,*cis*-12, were tested individually at concentrations similar to those in the CLA mixture, 42.6 and 43.6 µM, respectively. Only the *trans*-10,*cis*-12 isomer significantly reduced SCD activity (similar to the CLA mixture), while there was no effect by *cis*-9,*trans*-11 CLA. We also tested linoleic acid derivatives, 9-HODE and 9-HOO, which contained conjugated double bonds at the *trans*-10,*cis*-12 position. Both compounds were inhibitory to SCD, with 9-HOO (51.3 µM) being the more effective while 9-HODE (100 µM) was less active than *trans*-10,*cis*-12 CLA. Ethanol effect was ruled out since CLA treated as albumin complex or dissolved in ethanol showed the same inhibitory effect. To eliminate the possibility that *trans*-10,*cis*-12 CLA may have impacted on acyl CoA formation, additional experiments with acyl CoA synthetase (ACS) were performed. *trans*-10,*cis*-12 isomer was incubated



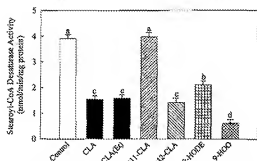


Fig. 1. Effects of different conjugated linoleic acid (CLA) isomers and linoleic acid derivatives on hepatic stearyl-CoA desaturase activity. Numbers are mean  $\pm$  S.E. ( $n=4-6$ , collected from three independent experiments). Means with different letters are significantly different ( $P<0.05$ ). The final concentrations were 100  $\mu$ M for CLA and 9-HODE, 42.6  $\mu$ M for *cis*-9, *trans*-11 CLA, 43.6  $\mu$ M for *trans*-10, *cis*-12 CLA, and 51.3  $\mu$ M for 9-HOO. CLA, *cis*-9, *trans*-11 CLA, and *trans*-10, *cis*-12 CLA were complexed with albumin. CLA(E), 9-HODE, and 9-HOO were dissolved in ethanol. All samples contained 100  $\mu$ M albumin, including control. CLA, conjugated linoleic acid; 9-HODE, 9-hydroxy-*trans*-10, *cis*-12 octadecadienoic acid; 9-HOO, 9-hydroperoxy-10,12-octadecadienoic acid.

with or without ACS (8 mU) for 5 min before adding microsomal fractions and then incubated for an additional 5 min. Inhibition by this isomer was not affected by addition of ACS (55% inhibition with ACS vs. 52.2% inhibition without ACS), indicating that formation of CoA was not the major process of inhibition by this isomer. The mean of two experiments showed a general linear trend on the inhibition

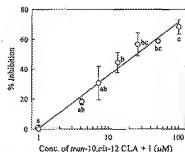


Fig. 2. Dose response of *trans*-10, *cis*-12 CLA on hepatic stearyl-CoA desaturase. Reported values are mean  $\pm$  S.E. ( $n=2-4$ , collected from two independent experiments). Means with different letters are significantly different ( $P<0.05$ ). To avoid zero values of log, the value of 1 was added to all data. CLA, conjugated linoleic acid.

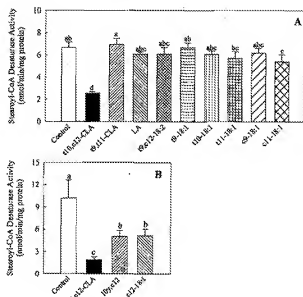


Fig. 3. Effects of fatty acids on hepatic stearyl-CoA desaturase activity. Numbers are mean  $\pm$  S.E. ( $n=4$ , collected from two independent experiments). Means with different letters are significantly different ( $P<0.05$ ). The final concentrations were 50  $\mu$ M for *trans*-10, *cis*-12 CLA, *trans*-9, *trans*-11 CLA, *cis*-9, *cis*-12 octadecadienoic acid (linoleic acid), *trans*-9, *trans*-11, *cis*-9, *cis*-11 and *cis*-12 octadecenoic acids, and 10y, *cis*-12, 31.7  $\mu$ M for *trans*-9, *cis*-12 octadecadienoic acid, and 49.4  $\mu$ M for *trans*-10 octadecenoic acid. CLA, conjugated linoleic acid; LA, linoleic acid; 10y, *cis*-12, *cis*-12-octadecen-10-ynoic acid.

of SCD over the dose of *trans*-10, *cis*-12 isomer of CLA tested (Fig. 2).

Fig. 3 shows the effects of other 18-carbon fatty acids including the *trans*, *trans* isomer of CLA. *trans*-9, *trans*-11 CLA, linoleic acid (*cis*-9, *cis*-12), and *trans*-9, *cis*-12 octadecadienoic acid did not have any effect on SCD. Neither did mono-unsaturated 18-carbon fatty acids, *trans*-9, *trans*-10, *trans*-11 (vaccenic acid), or *cis*-9 (oleic acid) octadecenoic acids. *cis*-11 and *cis*-12 octadecenoic acids inhibited this enzyme, but the effects were less than *trans*-10, *cis*-12 CLA (19% inhibition by *cis*-11 and 51% inhibition by *cis*-12 compared to 70% inhibition by the *trans*-10, *cis*-12 CLA isomer). 10y, *cis*-12 had similar effects as *cis*-12 octadecenoic acid (50% inhibition), but again was less effective than the *trans*-10, *cis*-12 isomer of CLA.

To test if there are any additional requirements for inhibition besides double bond location, we prepared

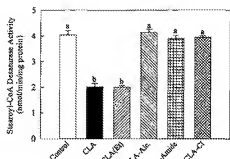


Fig. 4. Effects of CLA derivatives in hepatic stearyl-CoA desaturase activity. Numbers are mean  $\pm$  S.E. ( $n=4$ , collected from two independent experiments). Means with different letters are significantly different ( $P<0.001$ ). All final concentrations were 100  $\mu$ M. Testing materials including CLA(Et) were dissolved in ethanol, except CLA was complexed to albumin. All samples contained 100  $\mu$ M albumin, including control. CLA-Alc, CLA-alcohol form; CLA-Alc, CLA-aldehyde form; CLA-Cl, CLA-chloride form. See Section 2 for chemical names of the derivatives.

and tested alcohol, amide or chloride forms of CLA since these compounds have double bonds at the same location, but different functional groups. None of these derivatives showed any effect on SCD (Fig. 4).

To prove the specificity of *trans*-10,*cis*-12 CLA, we also tested arachidonic acid under the same experimental conditions. Polyunsaturated fatty acids, especially arachidonic acid, are known to reduce SCD activity by reducing mRNA expression (similar to previously reported effects of CLA). Fig. 5 shows that, again, the CLA mixture (100  $\mu$ M) significantly

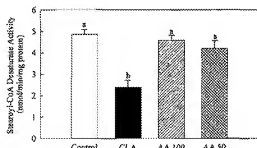


Fig. 5. Effects of arachidonic acid on hepatic stearyl-CoA desaturase activity. Numbers are mean  $\pm$  S.E. ( $n=4$ , collected from two independent experiments). Means with different letters are significantly different ( $P<0.05$ ). The final concentrations were 100  $\mu$ M for CLA and 50 and 100  $\mu$ M for arachidonic acid. CLA, conjugated linoleic acid; AA, arachidonic acid.

reduced SCD activity. However, unlike CLA, arachidonic acid (at 50 or 100  $\mu$ M) did not have any effect in this experiment.

#### 4. Discussion

Previously, Lee et al. [23] reported that feeding CLA mixture significantly reduced hepatic SCD activity in mice by reducing SCD mRNA expression. In that study, it was suggested that since the *cis*-9,*trans*-11 isomer had no effect, *trans*-10,*cis*-12 CLA (the other main isomer) inhibited SCD. Our results clearly support that the *trans*-10,*cis*-12 isomer causes inhibition of SCD. While Lee et al. [23] measured mRNA expression from CLA fed animals, in this study we added CLA directly to the incubation medium with microsomal fractions for a relatively short time period (15 min). Therefore, inhibition by CLA in this study was not due to reduced SCD mRNA levels, indicating that *trans*-10,*cis*-12 CLA directly inhibits SCD enzymatic activity. These results are consistent with the recent publication by Bretilion et al. [30], which showed inhibition of SCD enzyme activity by *trans*-10,*cis*-12, but not by *cis*-9,*trans*-11 isomer.

As a structural requirement of inhibition of SCD, Clarke and Jump [31] proposed that an inhibitory fatty acid must contain two double bonds positioned between carbons 9–10 and 12–13 of an 18-carbon fatty acid. In addition, at least one of these double bonds must be *cis*, but the other can be in the *trans* configuration. Since *trans*-10,*cis*-12 CLA inhibits SCD, but *cis*-9,*trans*-11 does not, it is possible that the *cis*-12 position, or alternatively the *n*-6 position, may be the more important double bond location. This is supported by the intermediate effect of *cis*-12 octadecenoic acid, but not *trans*-10 octadecenoic acid. The minimal effect of *cis*-11 octadecenoic acid may be the result of its similarity to the *cis*-12 isomer.

It is apparent from this study, however, that *trans*-10,*cis*-12 CLA has additional inhibitory mechanisms that are distinct from polyunsaturated fatty acid (PUFA) which are known inhibitors of SCD. For example, linoleic, linolenic, arachidonic and eicosapentaenoic acids have been reported to decrease SCD activity in liver or adipocytes by decreased SCD

mRNA expression either by decreasing transcription or by decreasing mRNA stability [32]. Even though Clarke and Jump [31] reported PUFA effect on transcription response is rapid, 24 h feeding was the shortest time tested in mice [33]. In fact, arachidonic, linoleic, and *trans*-9,*cis*-12 octadecadienoic acids, all of which are either known inhibitors or would be expected to inhibit SCD since they fit the inhibitor requirements described earlier, did not show any effect in our experiments. Because of the relatively short incubation time in our studies (15 min), the interaction between *trans*-10,*cis*-12 CLA and the enzyme itself is more important than any effects on SCD mRNA expression.

Hovik et al. [34] reported direct inhibition of 9 or 10 thia fatty acids on SCD activity. Stearoyl-CoA with sulfur at the 9th or 10th position (9- or 10-thia stearoyl-CoA) apparently does not interfere with binding to the SCD, but the fatty acid cannot be desaturated, thus causing inhibition of  $\Delta^9$  desaturation. However, it has been suggested that 3-thia fatty acid reduced the affinity to the enzyme but permits desaturation. *trans*-10,*cis*-12 CLA may have a similar mechanism to thia fatty acid on SCD activity.

Fig. 4 indicates that the acid forms (alternatively CoA form) of the *trans*-10,*cis*-12 CLA isomer, and also 9-HODE and 9-HOO, were active while other forms (alcohol, amide or chloride) were not. This may indicate that there are additional mechanisms or requirements, such as CoA form of fatty acid, that are necessary to interact with the enzyme.

Increased SCD activity in liver and adipose tissue has been reported in relation to obesity [35–37]. In addition to the effects in liver, recently Choi et al. [38] discovered that *trans*-10,*cis*-12 CLA decreased SCD1 mRNA expression and activity in 3T3-L1 adipocytes. Along with previous reports of decreased lipoprotein lipase (LPL) activity in adipocytes and increased fatty acid  $\beta$ -oxidation in muscle, the effects of *trans*-10,*cis*-12 CLA on SCD may help explain CLA's multiple mechanisms on body fat reduction. We previously suggested that CLA acts by mediating prostaglandin mechanisms; however, Sessler et al. [39] indicated that decreased SCD mRNA expression by PUFA is eicosanoid independent. These reports along with the results in this paper suggest that CLA may exert its effects through multiple mechanisms.

When the compounds shown in Figs. 3 and 4 are

tested for LPL activity in 3T3-L1 adipocytes, only CLA-Alc was inhibitory (33%), and it was less effective than the *trans*-10,*cis*-12 CLA isomer (68%, unpublished observation). Interestingly, Cook et al. [40] recently reported that CLA-amide, CLA-Alc and 10y,*cis*-12 reduced fat pad weights in mice, while *cis*-12 octadecenoic acid did not. The discrepancy between these results may be due to different model systems. Animals may convert CLA-amide, CLA-Alc or 10y,*cis*-12 to the *trans*-10,*cis*-12 CLA isomer, but this conversion may not occur in an in vitro assay, particularly since incubation time is limited. These results may indicate the specificity of *trans*-10,*cis*-12 CLA on inhibition of SCD activity. Alternatively, SCD may be unrelated with body fat regulation since *cis*-12 octadecenoic acid had an inhibitory effect on SCD, but no effect on body fat reduction in mice [40].

Based on these results, it is apparent that *trans*-10 and *cis*-12 double bond positions and perhaps conjugation may be key structures for directly inhibiting SCD. These results, along with Lee et al.'s previous study [23] indicate that *trans*-10,*cis*-12 CLA acts on SCD by direct inhibition as well as by decreasing SCD mRNA expression.

In summary, *trans*-10,*cis*-12 CLA directly inhibited hepatic SCD, while *cis*-9,*trans*-11 or *trans*-9,*trans*-11 CLA isomers did not. *Cis*-12 and *cis*-11 octadecenoic acids and 10y,*cis*-12 octadecadienoic acid also inhibited SCD, but to a lesser extent than *trans*-10,*cis*-12 CLA. Linoleic acid derivatives 9-HODE and 9-HOO showed effects similar to *trans*-10,*cis*-12 CLA, while other 18-carbon fatty acids (*trans*-9, *trans*-10, *trans*-11, or *cis*-9 octadecenoic acids, *trans*-9,*cis*-12 octadecadienoic acid, or linoleic acid), CLA derivatives (alcohol, amide and chloride), and arachidonic acid did not have any inhibitory effects using a microsomal enzyme activity assay. A *cis*-12 double bond appears to be a key structure for inhibiting SCD activity, especially when coupled with a *trans*-10 double bond, but not with a 9 double bond.

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# Effect of Conjugated Linoleic Acid on Body Composition in Mice

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**ABSTRACT:** The effects of conjugated linoleic acid (CLA) on body composition were investigated. ICR mice were fed a control diet containing 5.5% corn oil or a CLA-supplemented diet (5.0% corn oil plus 0.5% CLA). Mice fed CLA-supplemented diet exhibited 57% and 60% lower body fat and 5% and 14% increased lean body mass relative to controls ( $P < 0.05$ ). Total carnitine palmitoyltransferase activity was increased by dietary CLA supplementation in both fat pad and skeletal muscle; the differences were significant for fat pad of fed mice and skeletal muscle of fasted mice. In cultured 3T3-L1 adipocytes, CLA treatment ( $1 \times 10^{-4}$  M) significantly reduced heparin-releasable lipoprotein lipase activity (~66%) and the intracellular concentrations of triacylglyceride (~8%) and glycerol (~15%), but significantly increased free glycerol in the culture medium (+22%) compared to control ( $P < 0.05$ ). The effects of CLA on body composition appear to be due in part to reduced fat deposition and increased lipolysis in adipocytes, possibly coupled with enhanced fatty acid oxidation in both muscle cells and adipocytes. *Lipids* 32, 853–858 (1997).

CLA is the acronym for a group of positional and geometric isomers of conjugated dienoic derivatives of linoleic acid. They were identified as comprising an anticarcinogenic principal present in grilled ground beef and other food sources, especially dairy products (1–3). Synthetically prepared CLA inhibited carcinogen-induced neoplasia in several animal models (2,4,5) and reduced development of atherosclerosis in rabbits (6) and hamsters (7). CLA reduced tissue arachidonic acid levels and protected against the catabolic effects of endotoxin administration in mice, rats, and chickens without adversely affecting immune function (8,9). Recently it was established that CLA is a growth factor for young rats in that it enhanced body mass and feed efficiency (10). We now report the effects of CLA on body composition in mice.

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Abbreviations: CLA, conjugated linoleic acid; CPT, carnitine palmitoyltransferase; DMEM, Dulbecco's modified Eagle's medium; LPL, lipoprotein lipase.

## METHODS AND PROCEDURES

**Materials.** Linoleic acid was purchased from Nu-Chek-Prep Corporation (Elysian, MN); triolein, [9,10-<sup>3</sup>H(N)], (specific activity 12 Ci/mmol) from American Radiolabeled Chemicals Incorporated (St. Louis, MO); and [1-<sup>14</sup>C]linoleic acid (specific activity 55 mCi/mmol) from Amersham Life Science (Arlington Heights, IL). CLA was prepared as described (1).

**Animals and body composition analyses.** Weanling male ICR mice (experiment 1), 6-wk-old female (experiment 2) ICR mice, 6-wk-old male ICR mice [carnitine palmitoyltransferase (CPT) assay], feed ingredients (except for CLA), and semi-purified diet (TD94060, 99% basal mix) (Table 1) were purchased from Harlan Sprague-Dawley (Madison, WI). Diet was stored at -20°C until use. Mice were housed in a windowless room with a 12-h light-dark cycle in strict accordance to guidelines established by the Research Animal Resources Center of University of Wisconsin-Madison. Diet was fed *ad libitum*, freshly provided every day (experiment 1) or three times per week (experiment 2). After a 5-d adaptation period mice were randomly separated into groups and fed either control diet (5.5% corn oil) or CLA-containing diet

TABLE 1  
Diet Composition<sup>a</sup>

Ingredients	Treatment	
	Control (g/kg)	CLA (g/kg)
Sucrose	481	481
Casein, "Vitamin-Free" Test	210	210
Corn starch	150	150
D,L-Methionine	3	3
Corn oil	55	50
CLA	0	5
Cellulose	50	50
Mineral mix, AIN-76	35	35
Vitamin mix, AIN-76A	10	10
Calcium carbonate	4	4
Choline bitartrate	2	2
Ethoxyquin	0.1	0.1

<sup>a</sup>In experiment 2, diet was prepared as described in Reference 37, which contained the same amount of ingredients except 495 g sucrose and 200 g casein per kg diet, without additional calcium carbonate. CLA, conjugated linoleic acid.

(5.0% corn oil plus 0.5% CLA). For body composition analyses, animals were sacrificed, gut contents were removed (to obtain empty carcass weight), and the carcasses frozen at  $-20^{\circ}\text{C}$ . Frozen carcasses were chopped, ground, and freeze-dried to determine water content. Total nitrogen was analyzed by the Kjeldahl method (11). Carcass fat content was measured by extraction with diethyl ether overnight using a Soxhlet apparatus. Total ash content was determined by incineration ( $500$ – $600^{\circ}\text{C}$ , overnight).

**Cell culture.** 3T3-L1 preadipocytes were purchased from American Type Culture Collection (Rockville, MD) and cultured as described (12). Briefly, 3T3-L1 preadipocytes were grown to confluence at  $37^{\circ}\text{C}$  in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. At 2-d post-confluence (designated day 0) cell differentiation was induced with a mixture of methylisobutylxanthine ( $0.5$  mM), dexamethasone ( $0.25$   $\mu\text{M}$ ), and insulin ( $1$   $\mu\text{g}/\text{mL}$ ) in DMEM containing 10% fetal bovine serum. On day 2 this medium was replaced with medium containing 10% fetal bovine serum and insulin only. On day 4 and thereafter the medium consisted of DMEM plus 10% fetal bovine serum only; this medium was subsequently replaced with fresh medium at 2-d intervals.

Fatty acid-albumin complexes were prepared as described (13) with slight modifications and added to culture media 48 h prior to cell harvest. CLA ( $10$   $\mu\text{mol}$ ) or linoleic acid ( $10$   $\mu\text{mol}$ ), dissolved in  $0.1$  M KOH, was added to  $10$   $\mu\text{mol}$  bovine serum albumin solution in phosphate-buffered saline and incubated overnight at  $4^{\circ}\text{C}$ . The pH and volume were adjusted, respectively, to  $7.2$  and  $5.0$  mL (before filter sterilization) before use. Final concentrations of linoleic acid and CLA were  $100$   $\mu\text{M}$  except as indicated for the dose-response experiment. All dishes including control had final concentration of  $100$   $\mu\text{M}$  albumin.

Cell viability was tested using a number of parameters, as follows. The activities of citrate cleavage enzyme (14) and fatty acid synthetase (15) were determined. In addition 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co., St. Louis, MO) was used as described (16). Briefly, cells were cultured in a 96-well plate and treated with fatty acid-albumin complexes for 48 h as described above. After rinsing, cells were incubated with  $50$   $\mu\text{L}$  of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide ( $5$  mg/mL) in DMEM at  $37^{\circ}\text{C}$  for 4 h. Medium was then removed and  $200$   $\mu\text{L}$  of dimethyl sulfoxide added to each well to dissolve formazan crystals before quantifying at  $570$  nm with a Microplate Reader Model MR600 (Dynatech Industries, Inc., Alexandria, VA).

Free and esterified glycerol were determined using a Sigma Diagnostic Kit. Heparin-releasable lipoprotein lipase (LPL) activity (E.C. 3.1.1.34,  $10$  U heparin/mL media for 1 h at  $37^{\circ}\text{C}$ ) was measured as described (17). Recovery of free fatty acid was estimated at  $71\%$  by using [ $^{14}\text{C}$ ]linoleic acid. Protein was determined as described (18).

**CPT (E.C. 2.3.1.23) assay.** Mice were fed CLA-supplemented or control diet for 1 wk. Half the mice in each group were then fasted overnight prior to sacrifice by cervical dislo-

cation. Epididymal fat pad, skeletal muscle (hind leg), and liver were removed. Tissues were then homogenized in  $3$  vol (wt/vol) of  $0.25$  M sucrose,  $1$  mM EDTA,  $10$  mM Tris HCl, pH  $7.4$  at  $4^{\circ}\text{C}$ . Homogenates were centrifuged ( $700 \times g$  for  $10$  min); supernatant fluid was again centrifuged ( $12,000 \times g$  for  $15$  min). Pellets (mitochondrial fraction) were washed and stored at  $-70^{\circ}\text{C}$  after resuspension in  $70$  mM sucrose,  $220$  mM mannitol,  $2$  mM HEPES buffer, pH  $7.4$  and  $1$  mM EDTA. Total CPT activity was assayed by measuring the initial rates of CoASH formation (19).

**Statistical analyses.** Data in Figures 1 and 2 and Tables 2 and 3 were analyzed using the "2 independent sample *t*-test." Feed intake per cage was determined every day for experiment 1 (two mice per cage) or at 2–3-d intervals for experiment 2 (one mouse per cage). Feed intake data shown in Figures 1 and 2 are calculated under the assumption that in experiment 1 each mouse acted independently, and in experiment 2 mice consumed an equal amount of feed on each of the days constituting the feeding period. Two-way analysis of variance were performed on data (treatments and experiments) for Figure 3, Table 4, and the carcass protein analyses described in the Results section. Of major interest here are the comparisons among the treatments; these were computed using the Statistics Analysis System (SAS Users' Guide: Statistics, SAS Institute Inc., Cary, NC) with the General Linear Mean procedure and Least Square Means option. If the interaction between treatment and experiment was significant, this interaction was then used as the error term in the Least Square Means analysis.

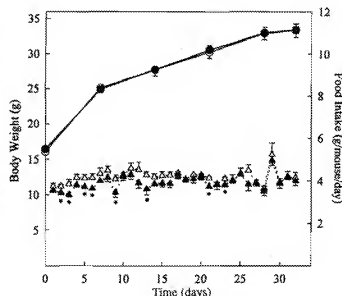


FIG. 1. Body weight (circle) and feed intake (triangle) from male mice fed control or conjugated linoleic acid (CLA)-supplemented diet (experiment 1). Four-week-old male mice were fed either control (open) or CLA (filled)-supplemented diet (0.5% of diet) for 32 d. Body weights ( $n = 8$ ) and feed intake were subjected to *t*-test ( $*P < 0.05$ ). Numbers are mean  $\pm$  SE.



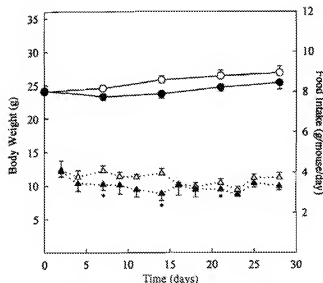


FIG. 2. Body weight (circle) and feed intake (triangle) from female mice fed control or CLA-supplemented diet (experiment 2). Seven-week-old female mice were fed either control (open) or CLA (filled) supplemented diet (0.5% of diet) for 28 d. Body weights and feed intake were subjected to *t*-test ( $^*P < 0.05$ ). Numbers are mean  $\pm$  SE ( $n = 6$ ). See Figure 1 for abbreviation.

## RESULTS

Figure 1 shows that male mice fed CLA-supplemented diet exhibited body weights that were indistinguishable from controls. By contrast, the data of Figure 2 indicate that female mice fed CLA appeared to exhibit slightly reduced body weight relative to controls, although the apparent reduction was not statistically significant. In both studies (Figs. 1 and 2) *ad libitum* feed intake for the CLA-fed animals appeared to be reduced at some experimental time points. We have previously shown that small differences in feed intake do not affect body composition (20).

Table 2 shows the effect of CLA supplementation on body composition in these animals. Relative to their respective controls, the percentage body fat in CLA-fed mice was reduced by 57% (males) and 60% (females). Hence, despite similar body weights (Figs. 1 and 2), dietary CLA supplementation resulted in significantly less body fat (Table 2).

By contrast, the percentages of whole body protein and

carcass water were significantly enhanced for CLA-fed mice in both experiments (Table 2). Because of the relatively small numbers of animals studied it is not possible to conclude from these data alone that CLA induced a significant increase in whole body protein accretion (whole body protein mass for controls and CLA-fed groups, respectively, were 5.7 and 6.0 g in experiment 1 and 4.4 and 4.6 g in experiment 2; in neither case was the difference significant). However, to date we have conducted 10 such mouse CLA feeding trials, only two of which are reported here in Figures 1 and 2 and Table 2. An analysis of the combined data from these 10 studies indicates that mice fed CLA-supplemented diets do in fact exhibit increased whole body protein relative to controls ( $P = 0.040$ ).

The mechanism of action of body composition changes is of considerable importance. Accordingly we studied the effect of CLA on total CPT, which is rate-limiting for fatty acid  $\beta$ -oxidation (Table 3). CPT activity was increased by dietary CLA supplementation in both fat pad and skeletal muscle; the differences were significant for fat pad of fed mice, and skeletal muscle of fasted mice. Liver CPT activity was not affected by CLA feeding.

Another key enzyme in lipid metabolism is adipocyte LPL, which hydrolyzes free fatty acids from circulating triacylglyceride; the fatty acids are then taken up by the adipocytes and re-esterified. We utilized 3T3-L1 adipocytes to measure LPL as well as fat mobilization. The amount of CLA added to the cell culture media was based on our determination that the mean CLA content of sera from rats fed diet supplemented with 0.5% CLA for 14 or 28 d is 72  $\mu$ M (range 23–120  $\mu$ M).

The data of Figure 3 show that supplementing the culture medium of fully differentiated 3T3-L1 adipocytes with CLA (but not linoleic acid) significantly reduced LPL activity. The inhibitory effect was linear between 20 and 200  $\mu$ M CLA (Fig. 4). The data indicate a threshold below 20  $\mu$ M where inhibition is not observed; the apparent enhancement at 5  $\mu$ M CLA is not understood and requires further study. In separate experiments to determine cell viability we found that incubating 3T3-L1 adipocytes for 2 d with 5 to 200  $\mu$ M CLA or linoleic acid did not change cell viability as measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (data not shown). We also found that incubating 3T3-L1 adipocytes for 2–3 d with CLA under similar conditions did not reduce citrate cleavage enzyme or fatty acid synthase ac-

TABLE 2  
CLA-Induced Changes in Body Composition<sup>a</sup>

Experiment		ECW (g) <sup>b</sup>	Fat (%)	Protein (%)	Water (%)	Ash (%)
1	Control	32.4 $\pm$ 1.1	10.13 $\pm$ 1.17	17.76 $\pm$ 0.30	66.3 $\pm$ 0.8	3.08 $\pm$ 0.14
	CLA	32.2 $\pm$ 0.8	4.34 $\pm$ 0.40 <sup>d</sup>	18.58 $\pm$ 0.14 <sup>d</sup>	70.9 $\pm$ 0.4 <sup>c</sup>	3.24 $\pm$ 0.05 <sup>c</sup>
2	Control	25.0 $\pm$ 0.9	18.68 $\pm$ 3.08	17.67 $\pm$ 0.61	58.3 $\pm$ 2.2	3.67 $\pm$ 0.15
	CLA	23.1 $\pm$ 1.0	7.47 $\pm$ 0.59 <sup>d</sup>	20.09 $\pm$ 0.24 <sup>d</sup>	66.2 $\pm$ 0.7 <sup>c</sup>	4.07 $\pm$ 0.09

<sup>a</sup>Study duration was 32 d (experiment 1) and 28 d (experiment 2). Reported values are means  $\pm$  SE ( $n = 6$  to 8).

<sup>b</sup>Empty carcass weight.

<sup>c</sup>Indicates CLA-fed mice are different from control, where <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$ , <sup>e</sup> $P < 0.001$ , and <sup>f</sup> $P < 0.0001$ . For other abbreviations, see Table 1.

**TABLE 3**  
Total Carnitine Palmitoyltransferase Activity in Fat Pad,  
Muscle, and Liver<sup>a</sup>

	Carnitine palmitoyltransferase (nmol/min/mg protein)		
	Fat pad	Muscle	Liver
Fasted			
Control	15.9 ± 1.7	16.8 ± 2.5	16.7 ± 1.6
CLA	18.4 ± 1.9	25.9 ± 2.1 <sup>c</sup>	17.7 ± 1.3
Fed			
Control	14.7 ± 2.1	11.7 ± 1.2	5.3 ± 0.9
CLA	22.4 ± 1.4 <sup>c</sup>	14.3 ± 1.7	5.3 ± 0.6

<sup>a</sup>Mice were fed control or CLA-supplemented diet (0.5%) for 1 wk. Reported values are means ± SE (n = 6).

<sup>b</sup>nmols L-carnitine exchange (Ref. 23).

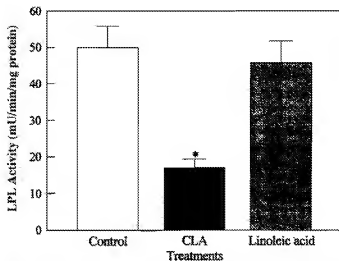
<sup>c</sup>P < 0.05 compared to control. For abbreviation, see Table 1.

activities (data not shown). Finally, there were no CLA- or linoleic acid-induced differences in total protein or heparin-released protein from the cells. Hence, the CLA effect reported here appears to be specific and not due to toxicity.

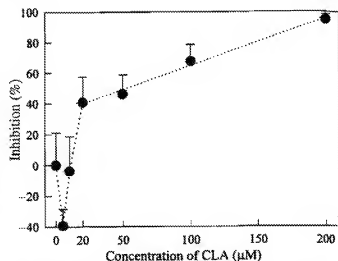
Table 4 shows the amount of esterified and free glycerol within the cells, and the free glycerol in the culture medium. The findings are consistent with Figures 3 and 4 in that CLA treatment resulted in a significant reduction in intracellular triacylglyceride (determined as esterified glycerol). However, the significant increase in free glycerol in the media indicates that CLA may have stimulated lipolysis as well. Linoleic acid also appears to have stimulated lipolysis in these cells, which is consistent with the findings of Takada *et al.* (21) that body fat was modestly reduced in rats fed gamma-linolenic acid (a metabolite of linoleic acid).

## DISCUSSION

The data of Table 2 establish that mice fed CLA-supplemented diets exhibited significant changes in body composition



**FIG. 3.** CLA inhibits lipoprotein lipase (LPL) activity in 3T3-L1 adipocytes. Reported values are means ± SE (n = 15–17, collected from four independent experiments). \*P < 0.05. See Figure 1 for abbreviation.



**FIG. 4.** Effect of CLA dose on LPL activity in 3T3-L1 adipocytes. Reported values are means ± SE of 3–4 culture dishes normalized against control. The  $r^2$  is 0.9901 for the data between 20 and 200 μM CLA. For abbreviations, see Figures 1 and 3.

tion relative to controls. This observation should be considered with regard to effects on the adipocyte (the principal site of fat storage) and the skeletal muscle cell (the principal site of fat oxidation).

The study described in Table 3 was conducted with tissues from control and CLA-fed mice. The findings are consistent with the interpretation that feeding mice a diet supplemented with CLA enhances fatty acid β-oxidation in skeletal muscle and fat pad, but not liver. These data (Table 3) may also explain at least in part our previous report (6) that rabbits fed CLA-supplemented diet exhibited reduced serum triglyceride levels.

The experiments described in Figures 3 and 4 and Table 4, which were conducted with 3T3-L1 adipocytes, indicate that CLA treatment reduced LPL activity while apparently enhancing lipolysis. If we assume that the *in vitro* experiments

**TABLE 4**  
Esterified and Free Glycerol in Cell Sonicates, and Free Glycerol  
in Culture Medium from Control and CLA-Treated 3T3-L1  
Adipocytes<sup>a</sup>

	Percentage of control		
	Control	CLA	Linoleic acid
Esterified glycerol			
in cell	100 <sup>a</sup>	91.7 ± 6.4 <sup>b</sup>	117.1 ± 8.0 <sup>c</sup>
Free glycerol			
in cell	100 <sup>a</sup>	84.9 ± 7.2 <sup>b</sup>	107.4 ± 5.9 <sup>a</sup>
Free glycerol			
in culture medium	100 <sup>a</sup>	122.0 ± 12.9 <sup>b</sup>	106.6 ± 2.2 <sup>b</sup>

<sup>a</sup>Cells were treated with 100 μM of CLA or linoleic acid for 48 h before assay. The means are for 10 independent experiments, normalized against their respective controls. Data (2–6 dishes per treatment in each experiment) were analyzed as log value with a two-way analysis of variance using fatty acid (as treatments) and experiment. If the interaction between treatment and experiment was significant, this interaction was used as the error term. Means with different superscript letters are significantly different (P < 0.05). For abbreviation, see Table 1.

with 3T3-L1 adipocytes (Figs. 3, 4, Table 4) reflect *in vivo* physiological changes, then these data taken together with the data of Table 3 appear to provide a framework for partially understanding the reduction in body fat that is evident in the data of Table 2. It should be noted that the data of Figures 3 and 4 and Table 4 support our very recent findings (22) that adipocytes isolated from rats fed CLA exhibited increased hormone-sensitive lipase activity and enhanced norepinephrine-induced lipolysis.

A possible explanation for the apparent enhancement of lean body mass (Table 2 and accompanying discussion of whole body protein accretion in the Results section) is less clear. This may be related to our previous reports that CLA protects against the catabolic effects of immune stimulation (8,9), which is modulated by prostaglandin  $E_2$  and mediated by interleukin-1 and tumor necrosis factor- $\alpha$ , both of which are linked to obesity (23-26). It is also possible that CLA affects pathways induced by anabolic hormones, perhaps in conjunction with an adaptive response by skeletal muscle necessitated by increased lipid substrate for  $\beta$ -oxidation. Further investigation is required.

There appear to be parallels between the effects of CLA reported and discussed herein and the reported effects of dietary fish oil. Like CLA, fish oil is reported to reduce fat pad size (27, but also see 28), prevent cachexia and body weight loss following immune stimulation (29,30), and reduce tissue arachidonic acid levels (31,32). The omega-3 fatty acids of fish oil modulate numerous biological and physiological effects that are linked to tumor necrosis factor- $\alpha$ , interleukin-1, and the eicosanoid pathways (30,33), indicating that the effects of CLA may also involve eicosanoid mechanism(s).

The isomers of CLA may be metabolized to one or more biologically active products which exert biological effect. For example CLA-derived eicosanoids could regulate the synthesis of arachidonic acid metabolites like prostaglandin  $E_2$ . Alternatively, CLA-derived eicosanoids might exhibit biological activity in their own right. These possibilities are at this time speculation, but they are also the focus of considerable ongoing research (8,9,34-36). Understanding the biochemical mechanism(s) of action of CLA will be crucial to fully utilizing the potential of this class of fatty acids in animal husbandry and human application.

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## Conjugated Linoleic Acid Is a Growth Factor for Rats as Shown by Enhanced Weight Gain and Improved Feed Efficiency<sup>1,2</sup>

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**ABSTRACT** We studied the effect of conjugated linoleic acid (CLA) on rat development and growth. Primigravid female Fischer rats were fed control or CLA-supplemented (0.25% or 0.5% CLA) diets during gestation and/or lactation. Conjugated linoleic acid was incorporated into milk fat and tissue lipids proportional to the level of CLA fed and the duration of CLA feeding. Conjugated linoleic acid was incorporated into fetal and neonatal tissues; it did not affect litter size nor induce apparent abnormalities. To the contrary, feeding CLA to the dams during gestation and lactation improved the postnatal body weight gain of pups ( $P < 0.05$ ), measured on d 10 of lactation. Pups that continued to receive the CLA-supplemented diet after weaning had significantly greater body weight gain and improved feed efficiency relative to control animals ( $P < 0.05$ ). *J. Nutr.* 124: 2344-2349, 1994.

### INDEXING KEY WORDS:

- conjugated linoleic acid • rats
- feed efficiency • growth enhancement

Conjugated linoleic acid (CLA) was first isolated from grilled ground beef and established as a potent cancer inhibitor with apparent antioxidant activity in several animal models [Ha et al. 1987 and 1990, Ip et al. 1991 and 1994]. Conjugated linoleic acid was then shown to reduce the catabolic effects of immune stimulation in mice, rats and chickens without adversely affecting immune function [Cook et al. 1993, Miller et al. 1994]. These findings indicate a complex mechanism of action for CLA that may involve effects on prostaglandin synthesis and signal transduction pathways. It should be noted that the catabolism of skeletal muscle, which is induced by immune stimulation, partitions energy away from other biological processes, including growth [Benson et al. 1993, Klasing and Austic 1984, Klasing et al. 1987].

Conjugated linoleic acid is present in virtually all foods [Chin et al. 1992], but the principal dietary sources are dairy products and other foods derived from ruminant animals [Bartlett and Chapman 1961, Chin et al. 1992]. Interestingly, turkey lipid contains CLA at levels similar to those of ruminant animals [Chin et al. 1992].

Kepler et al. [1966] identified the *cis*-9, *trans*-11 CLA isomer as an intermediate in the biohydrogenation of linoleic acid by the rumen bacterium *Butyrivibrio fibrisolvens*. Seemingly some of the CLA produced in this way escapes conversion to stearic acid, is absorbed from the digestive system, and is subsequently incorporated into tissue lipid [including milk phospholipid]. In this way CLA may be seen as an inherent component of cow's milk. Conjugated linoleic acid is also generated in the colon of conventional but not germ-free rats fed diets containing free linoleic acid [Chin et al. 1994]. This finding indicates that microorganisms in the digestive tracts of monogastric animals are able to synthesize CLA.

It follows from these observations that CLA may be a previously unrecognized growth factor for at least some animal species. The purpose of this study was to investigate this possibility. We found that feeding CLA to rat dams during gestation and lactation enhanced pup growth while producing no observed evidence of harm. Consumption of the CLA-fortified diets by the pups after weaning further extended the

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body weight gain and improved feed efficiency as well.

## MATERIALS AND METHODS

All protocols for animal use were approved by the Research Animal Resources Center of University of Wisconsin-Madison, and experimental procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (NRC 1985).

**Animals.** Two experiments were performed. In Experiment 1, 8-wk-old virgin female Fisher rats (Harlan Sprague Dawley, Indianapolis, IN) were used; in Experiment 2, 10-wk-old animals were used. Animals were maintained in individual cages at 20–22°C with 12-h light:dark photoperiods and fed a nonpurified diet (Ralston Purina, St. Louis, MO). They were transferred to separate cages for mating with males fed the same diet. Matings were confirmed by the presence of sperm in the vaginal smear. Day zero of gestation was defined as the first day of visible sperm in the vaginal smear.

Immediately after mating the females were removed and assigned to dietary groups. In Experiment 1, the animals were fed control diet or control diet fortified with 0.5 g CLA/100 g (Table 1). To ensure a homogenous mixture, CLA was mixed in corn oil before adding in basal diet. Diets were mixed weekly and stored under nitrogen at 4°C. Fresh diet was provided daily to each rat to minimize autooxidation. At d 20 of gestation, 10 rats each from the two treatment groups were killed by carbon dioxide suffocation; liver, mammary gland, skeletal muscle and abdominal adipose tissues were then removed. Fetuses were removed, weighed and examined visually for abnormalities. Maternal and fetal tissues were stored at –80°C until analyzed. The remaining rats (see footnotes to Tables 4 and 5) were allowed to deliver at term, and the day of parturition was designated as day zero of lactation.

The same diets were used in Experiment 2, with the addition of a third group fed control diet fortified with 0.25 g CLA/100 g. A fourth group received the control diet during gestation and the 0.5% CLA diet during lactation. Pups were weighed on d 10 and weaned on d 22 of lactation. Immediately after weaning, male and female pups were housed in individual cages and fed for 8 and 10 wk, respectively, the same diet their mothers had received. Body weights and food intakes were determined weekly.

**Milk collection and analysis.** Milk was collected on d 10 of lactation between 1200 and 1400 h, to minimize possible diurnal variations in milk composition (Picciano 1978). Dams were separated from their litters 4 h before milking; longer periods of separation can affect milk composition (Keen et al. 1980). Dams were anesthetized with sodium pentobarbital (Butler Company, Columbus, OH). Oxy-

TABLE 1

*Diet composition<sup>1</sup>*

Component	Amount g/kg diet
Casein, vitamin-free test	216.5
DL-Methionine	3.1
Sucrose	495.83
Cornstarch	154.64
Corn oil	25.78
Cellulose	51.55
Mineral mix, AIN-76	36.09
Calcium carbonate	4.13
Vitamin mix, AIN-76A	10.31
Choline bitartrate	2.07

<sup>1</sup>To facilitate the mixing of conjugated linoleic acid (CLA) into the diet, only 25.78 g corn oil/kg diet was included in the basal diet. Additional corn oil (25 g/kg diet) was used as a carrier for CLA. The final compositions of the diets were as follows (g/kg): 0.5% CLA, 50.78 g corn oil + 5 g CLA; 0.25% CLA, 53.28 g corn oil + 2.5 g CLA; control, 55.78 g corn oil.

tocin (1.2 USP units) (Fermentia Animal Health Company, Kansas City, MO) was injected intraperitoneally to stimulate milk flow. Milk was drawn by applying intermittent mild suction to each nipple through a Tygon plastic tube (Norton, Akron, OH) and collected in plastic tubes. Milk was not pooled; rather, milk from each dam was portioned into a number of plastic vials and stored frozen (–80°C).

Total protein in milk was determined by the BCA protein assay kit (Pierce Chemical, Rockford, IL). Total milk lipid was extracted as described by Supelco Inc. with slight modification due to limited sample size. To a 16-mL screw-capped tube the following was added: 0.5 mL of rat milk, 1 mL of ethanol, 0.3 mL of 16.44 mol/L ammonium hydroxide, 2.5 mL of petroleum ether, and 2.5 mL of diethyl ether. Samples were shaken for 5 min and allowed to stand for 20 min. The bottom phase was discarded; the ether layer was washed with water, filtered into a tared screw-capped test tube, and then dried under nitrogen. Net fat was weighed, capped under argon gas, and stored at –80°C.

**Conjugated linoleic acid.** Conjugated linoleic acid was synthesized as previously described (Chin et al. 1992). Quantification of total CLA methyl ester in milk fat and animal tissues was performed by HPLC (Chin et al. 1992). Gas chromatographic analyses of CLA isomers were conducted as described (Ha et al. 1989).

**Nucleic acid analysis.** Nucleic acids were extracted from the mammary gland (Ogur and Rosen 1950). RNA was quantified (Ceriotti 1955) using baker's yeast RNA (Sigma Chemical, St. Louis, MO) as the standard. DNA was quantified (Hubbard et al. 1970)

using calf thymus DNA (Sigma Chemical) as the standard.

**Statistical analyses.** Data were analyzed by ANOVA using the general linear model procedure of the SAS statistical software (SAS Institute, Cary, NC). Significant differences among means were determined using the Tukey's comparisons (Steel and Torrie 1980). Litter size was tested for a potential covariate between pup weight and milk protein, but no significant effect was observed ( $P > 0.12$ ). Therefore only one-way analysis was performed on pup body weight and milk protein. Differences were considered significant at  $P < 0.05$ .

## RESULTS

**Experiment 1.** Consumption of CLA during gestation did not affect the food intake or body weights of the dams (Table 2). Mammary gland weights and DNA and RNA concentrations were comparable for CLA-fed and control animals, indicating that CLA did not directly affect mammary gland development. Liver weights for the two groups were also similar.

Litter sizes (0.5% CLA, 9.3; control, 9.2) and fetal body weights (0.5% CLA,  $3.27 \pm 0.05$  g; control,  $3.27 \pm 0.28$  g) at 20 d of gestation were unaffected by CLA consumption. Fetal liver (0.5% CLA,  $0.22 \pm 0.01$  g; control  $0.23 \pm 0.02$  g) and brain weights (0.5% CLA,  $0.16 \pm 0.01$  g; control,  $0.15 \pm 0.01$  g) were similar in the two groups. There was no gross visible evidence of fetal abnormality.

TABLE 2

*Lack of effect of consumption of a diet containing 0.5 g conjugated linoleic acid/100 g diet (0.5% CLA) on mammary gland development in rats<sup>1,2</sup>*

	Dietary group	
	Control	0.5% CLA
Total food intake, g	251.1 $\pm$ 3.4	247.7 $\pm$ 6.33
Maternal body weight, g	226.3 $\pm$ 4.14	221.9 $\pm$ 2.84
Mammary gland weight, g	6.70 $\pm$ 0.32	6.69 $\pm$ 0.36
Net body weight of dams, g	196.1 $\pm$ 4.42	192.0 $\pm$ 2.19
Mammary gland weight/ 100 g net body wt, g	3.43 $\pm$ 0.19	3.49 $\pm$ 0.19
Liver weight, g	12.7 $\pm$ 0.25	13.1 $\pm$ 0.72
RNA <sup>3</sup>	73.8 $\pm$ 5.11	77.6 $\pm$ 5.60
DNA <sup>3</sup>	34.2 $\pm$ 4.16	37.8 $\pm$ 3.37
RNA:DNA ratio	2.45 $\pm$ 0.34	2.16 $\pm$ 0.18

<sup>1</sup>Values are means  $\pm$  SE. There were 9 dams in the control group and 10 dams in the CLA-fed group.

<sup>2</sup>Body weight and mammary gland weight were measured on d 20 of gestation. Net body weight was calculated as maternal body weight minus fetal body weight.

<sup>3</sup>Milligrams per gram of dry fat-free mammary gland.

TABLE 3

*Maternal and fetal tissue CLA concentrations in rats fed a diet containing 0.5 g conjugated linoleic acid/100 g diet (0.5% CLA)<sup>1,2</sup>*

	Dietary group	
	Control	0.5% CLA
	$\mu\text{mol CLA/g tissue lipid}$	
Maternal liver	0.53 $\pm$ 0.18	12.0 $\pm$ 1.03
Maternal muscle	0.78 $\pm$ 0.11	15.9 $\pm$ 1.36
Maternal mammary gland	1.21 $\pm$ 0.11	29.7 $\pm$ 1.43
Fetal liver	0.21 $\pm$ 0.03	4.96 $\pm$ 0.64

<sup>1</sup>Values are means  $\pm$  SE. There were 9 dams in the control group and 10 dams in the CLA-fed group.

<sup>2</sup>Tissues were collected on d 20 of gestation. Livers from a litter were pooled together for fat extraction. Litter sizes were comparable (control group, 9.3 pups per dam; CLA-fed group, 9.2 pups per dam).

Tissue from control dams and fetuses contained low but detectable amounts of CLA (Table 3). In contrast, the CLA concentration in comparable tissue from animals fed the diet containing 0.5% CLA was substantially elevated. Conjugated linoleic acid was incorporated into milk fat of dams fed the CLA-supplemented diet (Table 4). Pups receiving CLA during gestation and lactation were significantly heavier than pups from control dams ( $P < 0.036$ ).

**Experiment 2.** The CLA concentration of milk increased in direct proportion to dietary CLA level (Table 5). Pup development and survival were not affected by dietary treatment. However, for the group fed the diet containing 0.5% CLA, pup weight on d 10 of lactation was significantly higher than for controls or for the group fed the diet containing 0.25% CLA ( $P < 0.03$ ). Pups from dams fed the 0.5% CLA-supplemented diet during lactation only were intermediate in weight.

TABLE 4

*Effect of consumption of a diet containing 0.5 g conjugated linoleic acid/100 g diet (0.5% CLA) on rat pup weight, litter size and CLA concentration in milk<sup>1,2</sup>*

	Dietary group	
	Control	0.5% CLA
Mean pup weight, g	9.61 $\pm$ 0.4	10.7 $\pm$ 0.5*
Litter size	6.81	6.92
CLA in milk, $\mu\text{mol/g milk fat}$	1.68 $\pm$ 0.21	46.7 $\pm$ 3.0

<sup>1</sup>Values are means  $\pm$  SE. There were 11 dams in the control group and 13 dams in the CLA-fed group. \* $P < 0.036$  as determined by ANOVA.

<sup>2</sup>Pups were on d 10 of lactation. Approximately 3 mL of milk was collected on d 10 of lactation.

TABLE 5

Effects of consumption of diets containing conjugated linoleic acid (CLA) during gestation and lactation, or during lactation only<sup>1,2</sup>

	Dietary group <sup>3</sup>			
	Control (n = 15)	0.25, 0.25 (n = 7)	0.5, 0.5 (n = 17)	0, 0.5 (n = 10)
CLA in milk, $\mu\text{mol/g fat}$	0.96 $\pm$ 0.03	22.3 $\pm$ 1.43	46.8 $\pm$ 1.43	42.5 $\pm$ 0.71
Litter size	10.4	9.9	10.3	9.7
Mean pup weight, g	12.8 $\pm$ 0.4 <sup>a</sup>	12.8 $\pm$ 0.6 <sup>a</sup>	14.0 $\pm$ 0.3 <sup>b</sup>	13.5 $\pm$ 0.3 <sup>ab</sup>

<sup>1</sup>Values are means  $\pm$  SE. Numbers in parentheses indicate number of dams. Values for mean pup weight that do not share a common superscript are significantly different ( $P < 0.05$ , Tukey's test).

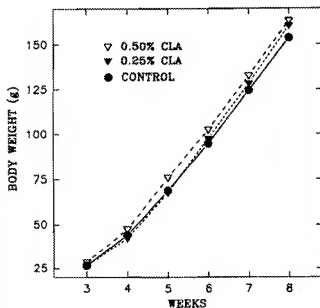
<sup>2</sup>Pups were weighed on d 10 of lactation for litters of nine to 11 pups. Approximately 3 mL of milk was collected on d 10 of lactation.

<sup>3</sup>The first and second numbers indicate the dietary percentages of CLA fed during gestation and lactation, respectively.

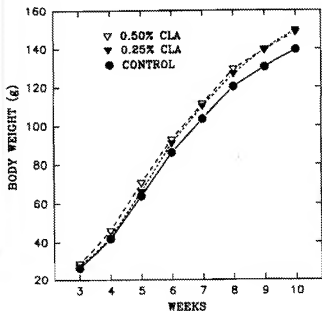
Figure 1 depicts body weights and food intakes, from weaning to 8 wk of age, for the male pups from the three treatment groups. Animals fed the 0.5% CLA-supplemented diet exhibited a small but consistent and statistically significant enhancement in body weight compared with control rats, whereas animals fed the 0.25% CLA-supplemented diet were intermediate in weight. Food intake over this period was not affected by treatment (Table 6). Feed efficiency (gram of body weight gain per gram of food

consumed over this time period) was of the order 0.5% CLA-fed group > 0.25% CLA-fed group > control group. The difference in feed efficiency values for the 0.5% CLA-fed group vs. the control group was significant ( $P < 0.05$ ). Hence, supplementing the diet with 0.5% CLA resulted not only in greater body size but also in greater efficiency of feed utilization.

Female pups exhibited similar characteristics (Fig. 2, Table 7). Feed efficiencies for both the 0.5% and 0.25% CLA-fed groups were significantly higher than for the control group ( $P < 0.05$ ).



**FIGURE 1** Body weights of male rats fed control or treatment diets from 3 to 8 wk of age. Values are means  $\pm$  SE at each week for rats fed the control diet ( $n = 43$  rats), the diet containing 0.25% conjugated linoleic acid (CLA) ( $n = 34$  rats), and the diet containing 0.5% CLA ( $n = 59$  rats). SE bars are within the size of symbol. Final body weights for both the 0.5% CLA-fed and the 0.25% CLA-fed groups are significantly higher ( $P < 0.05$ ) than that of the control group (Tukey's test).



**FIGURE 2** Body weights of female rats fed control or treatment diets from 3 to 10 wk of age. Values are means  $\pm$  SE at each week for rats fed the control diet ( $n = 47$  rats), the diet containing 0.25% conjugated linoleic acid (CLA) ( $n = 28$  rats), and the diet containing 0.5% CLA ( $n = 60$  rats). SE bars are within the size of symbol. Final body weights for both the 0.5% CLA-fed and the 0.25% CLA-fed groups are significantly higher ( $P < 0.05$ ) than that of the control group (Tukey's test).



TABLE 6

Effect of consumption of diets containing conjugated linoleic acid (CLA) on growth performance of male rats (5 wk after weaning)<sup>1,2</sup>

	Dietary group		
	Control (n = 43)	0.25% CLA (n = 34)	0.5% CLA (n = 59)
Total body weight gain, g	126.8 ± 1.34*	132.4 ± 1.79 <sup>b</sup>	134.9 ± 0.81 <sup>b</sup>
Total food intake, g	350.3 ± 2.86	359.5 ± 3.55	354.8 ± 2.44
Feed efficiency <sup>3</sup>	0.362 ± 0.01*	0.374 ± 0.01 <sup>ab</sup>	0.381 ± 0.01 <sup>b</sup>

<sup>1</sup>Values are means ± SE, with the number of rats in parentheses. Within a row, values that do not share a common superscript are significantly different ( $P < 0.05$ , Tukey's test).

<sup>2</sup>Rats were weaned (on d 22 of lactation) from dams that had been fed control or CLA-supplemented diet during gestation and lactation. Immediately after weaning, rats were housed in individual cages and fed treatment diets until 8 wk old.

<sup>3</sup>Feed efficiency = gram of body weight gain per gram of food intake.

We also studied the effect of CLA consumption on the protein concentration of rat milk. In Experiment 1 there was more protein in milk from dams fed diet containing CLA (mice fed diet supplemented with 0.5% CLA, 114.1 ± 4.3 g protein/L; controls, 95.15 ± 3.0 g protein/L,  $P < 0.006$ ). However, in Experiment 2 there were no differences among groups (mice fed diet supplemented with 0.5% CLA, 98.6 ± 1.7 g protein/L; mice fed diet supplemented with 0.25% CLA, 88.1 ± 4.5 g protein/L; controls, 96.5 ± 2.1 g protein/L). Hence, the effects reported here were not related to difference in milk protein concentration.

## DISCUSSION

Conjugated linoleic acid is detectable in virtually every animal-derived food examined [Chin et al. 1992] and is consistently found in the tissues of rodents irrespective of diet [Table 4] [Chin et al. 1994, Ha et

al. 1990, Ip et al. 1991]. Conjugated linoleic acid is elevated in meat derived from ruminant animals and seems to be an inherent component of cow's milk [Bartlett and Chapman 1961, Chin et al. 1992]. In addition, elevated CLA levels occur in fresh and processed turkey meat [Chin et al. 1992] and in tissues of conventional but not germ-free rats fed diets containing free linoleic acid [Chin et al. 1994].

Conjugated linoleic acid displays a number of biological activities that may be regarded as beneficial. Conjugated linoleic acid inhibits chemically induced neoplasia at some sites in rats and mice [Ha et al. 1987 and 1990, Ip et al. 1991 and 1994] and seems to reduce blood LDL concentrations and inhibit the development of atherosclerosis in rabbits [Lee et al. 1994] and hamsters [Nicolosi et al. 1993] fed atherogenic diets. Conjugated linoleic acid reduces the catabolic response induced by immune stimulation in mice, rats and chickens without adversely affecting immune function [Cook et al. 1993, Miller et al.

TABLE 7

Effect of consumption of diets containing conjugated linoleic acid (CLA) on growth performance of female rats (7 wk after weaning)<sup>1,2</sup>

	Dietary group		
	Control (n = 47)	0.25% CLA (n = 28)	0.5% CLA (n = 60)
Total body weight gain, g	113.6 ± 1.07*	120.7 ± 0.78 <sup>b</sup>	122.8 ± 1.43 <sup>b</sup>
Total food intake, g	475.7 ± 4.83	473.4 ± 3.69	483.1 ± 5.91
Feed efficiency <sup>3</sup>	0.239 ± 0.01*	0.255 ± 0.01 <sup>b</sup>	0.254 ± 0.01 <sup>b</sup>

<sup>1</sup>Values are means ± SE, with the number of rats in parentheses. Within a row, values that do not share a common superscript are significantly different ( $P < 0.05$ , Tukey's test).

<sup>2</sup>Rats were weaned (d 22 of lactation) from dams that had been fed control or CLA diet during gestation and lactation. Immediately after weaning, rats were housed in individual cages and fed treatment diets until 10 wk old.

<sup>3</sup>Feed efficiency = gram of body weight gain per gram of food intake.

1994]. This response is cytokine-mediated and regulated by prostaglandin  $E_2$  synthesis. It is possible that these seemingly disparate effects of CLA are mechanistically related through effects on prostaglandin  $E_2$  metabolism and possibly also signal transduction pathways; the observation that CLA exhibits antioxidant activity in vitro and in vivo, especially in tissues (e.g., mammary gland) in which it is also an anticarcinogen [Ha et al. 1990, Ip et al. 1991] may also be mechanistically important.

Fish oil reduces the catabolic response induced by immune stimulation [Meydani 1992], but in mice and chickens CLA seems much more effective in this regard [Cook et al. 1993, Miller et al. 1994]. Bacteria, dust and related materials, which occur naturally in virtually all environments, serve as immune stimulants. The catabolism of skeletal muscle that follows immune stimulation partitions energy away from other biological processes. Reducing this response enhances growth and improves feed efficiency [Benson et al. 1993, Klasing and Austic 1984, Klasing et al. 1987].

Conjugated linoleic acid may be a previously unrecognized nutrient that functions to modulate the potentially devastating negative effects of immune stimulation. Growth promotion would be an expected reflection of such a physiological activity.

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FEEDING CONJUGATED LINOLEIC ACID TO ANIMALS PARTIALLY  
OVERCOMES CATABOLIC RESPONSES DUE TO ENDOTOXIN INJECTION

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**Summary:** The ability of conjugated linoleic acid to prevent endotoxin-induced growth suppression was examined. Mice fed a basal diet or diet with 0.5% fish oil lost twice as much body weight after endotoxin injection than mice fed conjugated linoleic acid. By 72 hours post injection, mice fed conjugated linoleic acid had body weights similar to vehicle injected controls; however, body weights of basal and fish oil fed mice injected with endotoxin were reduced. Conjugated linoleic acid prevented anorexia from endotoxin injection. Splenocyte blastogenesis was increased by conjugated linoleic acid.

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Recently, nonessential fatty acids have been shown to modulate acute inflammatory and immune responses possibly by changing cellular fatty acid profiles (1). The ability of two major fatty acid components of fish oil, eicosapentaenoate (EPA) and docosahexaenoate (DHA), to modulate the synthesis of arachidonate and its metabolites was recognized as one mechanism of immune regulation by fatty acids. EPA has been shown to depress arachidonic acid derived prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) synthesis in peritoneal leukocytes from rats (2). Unlike corn oil-fed rats, fish oil-fed rats did not exhibit the characteristic depression in food intake when injected with interleukin-1 (IL-1) (3). This phenomena has been partially attributed to the ability of fish oil to block or alter the cyclooxygenase pathway thereby decreasing PGE<sub>2</sub> synthesis and altering the ability of PGE<sub>2</sub> to negatively feed back on IL-1 synthesis.

CLA (conjugated dienoic derivative of linoleic acid) was shown to be a naturally occurring substance in food which has an anticarcinogenic effect on dimethylbenz(a)anthracene induced

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mammary tumors (4). Recent studies (5) have shown that CLA decreases arachidonic acid content of select tissues. It was postulated that like fish oil, CLA may exert an effect on the immune system by altering product formation of either the cyclooxygenase or lipoxygenase pathway. The purpose of the following investigation was to determine the effect of CLA on endotoxin induced growth inhibition and food intake depression. Fatty acid levels in tissue and *in vitro* lymphocyte proliferation was examined.

#### Materials and methods

Preparation of CLA and purity determination: CLA was prepared from linoleic acid by alkali isomerization as previously described (6, 7). Purity was determined to exceed 97%. CLA was stored in an argon atmosphere at -20°C.

Tissue fatty acid determination: Tissue fatty acid levels were determined by the following procedure. Total fat was extracted by chloroform:methanol (2:1, v/v) as described by Folch et al (8). Fatty acid methyl esters were prepared by reaction with 4% HCL in methanol for 20 minutes at 60 C (9) and extracted with hexane. Tridecanoic acid methyl ester was used as an internal standard and fatty acid methyl esters were identified by comparison with standards using gas chromatography (Hewlett Packard 5890 series II) (7).

Mouse experimental protocol: Three-week old mixed-sex mice (U.W. Madison Dairy Science Dept. outbred colony) (4 per group and 3 groups per dietary treatment) were fed a semi-purified basal diet (containing 2.5% linoleic acid) (Harlan Teklad, Madison, WI) or the basal diet containing either 0.5% added Menhaden fish oil with 25-30% omega-3 fatty acids as triglycerides (Sigma Chemical Co., St. Louis, MO) or 0.5% added CLA. All fatty acids were mixed into the basal diet following the protocol of Frische and Johnston (10) to minimize autooxidation. At the end of 15 consecutive feeding days, mice were weighed and i.p. injected with lipopolysaccharide (E. Coli 055:B5, Sigma Chemical Co., St. Louis, MO) at 1 mg/kg body weight in sterile HEPES buffer (Sigma Chemical Co., St. Louis, MO) or HEPES buffer alone. All mice were weighed 3, 8, 24, 48 and 72 hours post injection, and feed intake was determined at 3, 8 and 24 hours post injection.

At sacrifice, spleens were excised and single cell suspensions were made by teasing the spleen apart in calcium and magnesium free buffer (pH 7.0) and passing through an 18-gauge needle. Cells were then centrifuged through Histopaque 1077 (density 1.077) (Sigma Chemical Co., St. Louis, MO) and the buffy layer containing mononuclear cells was collected. Mononuclear cells were counted on a hemocytometer and tested for viability using trypan blue exclusion. Blastogenesis was measured following the procedure of Hughes et al (11). Results are expressed as a DPM index (disintegrations per minute in stimulated sample cells/ DPM in unstimulated sample cells).

Rat and chick experimental protocol: CLA (0.5% mixed into a semi-purified rat diet (Harlan Teklad, Madison, WI) or a standard chick mash) was fed to 20 rats (Harlan Sprague Dawley, Madison, WI) and 24 chicks (Leghorn X New Hampshire, Poultry Research Laboratory, Madison, WI) for 14 and 7 consecutive days, respectively. Control animals were fed their respective basal diets. After the feeding period, animals were injected i.p. with

endotoxin (see mouse protocol) and body weight losses due to the injection were monitored.

**Statistics:** Data were subjected to analysis of variance using the general linear models procedure of the Statistical Analysis System (12). Mean differences were determined using the least significant difference method.

### Results

Feeding CLA to mice prevented the severe body weight loss caused by endotoxin injection as observed in mice fed the basal diet (figure 1). Fish oil (.5% of diet) was ineffective in preventing endotoxin-induced weight loss. Within the first 12 hours after endotoxin injection, body weight loss was equal among treatment groups. However, by 24 hours after injection, body weight losses in basal and fish oil fed mice were twice that of CLA fed mice. Return to pre-injection body weight also appeared to be faster in the groups fed CLA. In a similar manner, twenty-four hours post endotoxin injection, the reduction in body weight gain in CLA fed chicks (+1 g/24 hrs vs. +3 g/24 hrs for noninjected controls) and rats (-8 g/24 hrs vs +9 g/24 hrs for noninjected controls) was significantly less than endotoxin injected chicks (-6 g/24 hrs) and rats (-15 g/24 hrs) fed their respective basal diets.

Mice injected with buffer consumed significantly more feed at all time periods measured than mice fed either the basal diet or basal diet plus fish oil and injected with endotoxin (figure 2). The latter two groups consumed no feed during the 24 hour period after injection. However, mice fed CLA also consumed significantly more feed than mice fed fish oil or the basal diet respectively. Mice from all treatments which were injected with

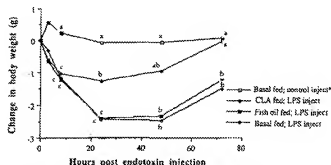


Figure 1. Change in body weight due to endotoxin injection in mice consuming diets containing CLA or fish oil.

\*Not different from CLA or fish oil fed and control inject.

abc Means with different letters are significantly different ( $p < .05$ ).

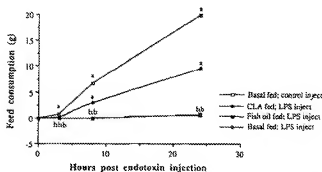


Figure 2. Effect of endotoxin injection on cumulative feed intake in mice.

ab Means with different letters are significantly different ( $p < .05$ ).

buffer had feed consumptions and body weight gains which did not differ.

Mice fed CLA had a 1.5 fold increase in PHA-P induced spleen lymphocyte blastogenesis over basal fed mice (figure 3). CLA fed mice also displayed greater responses than fish oil fed mice, although this difference was not significant.

The muscle content of CLA was increased in rats (6.25 vs. .94 mg/g fat;  $p < .05$ ) and chicks (4.07 vs. not detectable;  $p < .05$ ) fed CLA relative to basal fed controls. Muscle arachidonic acid levels were decreased in CLA fed animals relative to the basal fed control animals (60.8 vs. 39.9 mg/g fat in rats and 43.6 vs. 38.8 mg/g fat in chicks).

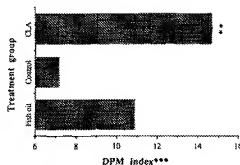


Figure 3. Effect of feeding fish oil or CLA on PHA-P induced spleen lymphocyte blastogenesis in mice.

\*PHA-P level = .78 $\mu$ g/500,000 cells.

\*\*Significantly greater than control ( $p < .08$ ).

\*\*\*DPM sample/DPM control.

### Discussion

Klasing et al (13) showed that endotoxin induced weight loss was the result of not only reduced food intake, but also reduced fractional protein synthesis rate in the gastrocnemius and increased skeletal muscle degradation. Likewise, the ability of animals fed CLA to maintain body weight gain during endotoxin challenge may be due to factors other than maintenance of feed intake. Endotoxin injection has been shown to enhance IL-1 production, and semi-purified IL-1 was capable of decreasing rate of gain and feed intake to the same extent as endotoxin injection (13). Recently it has been demonstrated that feed intake depression associated with immune stimulation via IL-1 injection can be prevented by feeding 8% fish oil containing omega-3 fatty acids (3). Fish oils added in human diets increased cell membrane EPA (an n-3 fatty acid) and decreased cell membrane arachidonic acid (an n-6 fatty acid). The change in cell membrane fatty acids was thought to alter prostaglandin production and thus prevented body weight loss due to IL-1 injection (3). It was also thought that a depression of PGE<sub>2</sub> at the skeletal muscle level depressed protein degradation during IL-1 stimulation (14) since IL-1 was shown to enhance muscle catabolism by inducing PGE<sub>2</sub> production (15). It is possible that CLA acted through a similar mechanism and thereby altered cell membrane fatty acids and depressed PGE<sub>2</sub> production. Indeed CLA decreased skeletal muscle arachidonic acid levels. Thus animals maintained growth during immune stimulation.

Michel et al (16) recently reported that CLA stimulated mitogen-induced lymphocyte blastogenesis, cytotoxic activity and macrophage killing ability. In agreement with Michel and co-workers, we also have shown that CLA enhances PHA-P induced lymphocyte blastogenesis relative to basal fed controls.

### Acknowledgment

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**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Application of: Jan Wadstein *et al.*  
Serial No.: 09/410,484  
Filed: 09/30/99  
Entitled: **Method Of Treating Hypertension And Reducing Serum Lipase Activity**  
Group No.: 1614  
Examiner: Webman

**Declaration of Asgeir Sæbo**

Assistant Commissioner for Patents  
Washington, D.C. 20231

<b>CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.8(a)(1)(i)(A)</b>	
<small>I hereby certify that this correspondence (along with any referred to as being attached or enclosed) is, on the date shown below, being deposited with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.</small>	
Dated <u>4-25-07</u>	By: 

I, Asgeir Sæbo, state as follows:

1. My present position is Research Director, Natural ASA.
2. It is my understanding that the Examiner has stated the following in the Office Action dated January 25, 2007:

First, Kawamura *et al.* provide a nexus teaching between weight loss in hypertensive patients and lowering of blood pressure. It remains obvious to one of ordinary skill in the art that the method of Cook *et al.* can lower blood pressure via weight loss. The Examiner did not assert that CLA would have a positive or negative on hypertension like ephedrine as submitted by Applicant. The Examiner asserts that weight loss is directly related to lowering of blood pressure, as supported by Kawamura *et al.*, and CLA can be used to effect weight loss as taught by Cook *et al.* Secondly, one of ordinary skill in the art would have a reasonable expectation of success because of the nexus teaching of Kawamura *et al.*

3. When a biologically active agent, such as CLA, is administered to a subject there can be a variety of effects. Just because CLA causes weight loss does not also mean that it would reduce hypertension. A person of skill in the art would not reasonable expect CLA to reduce hypertension for two reasons.

4. First, CLA has been shown to elevate the level of F2-isoprostane. Taylor et al., *Conjugated Linoleic Acid Impairs Endothelial Function, Arteriosclerosis, Thrombosis, and Vascular Biology* 26(2), 307-312 (2006)(attached at Tab 1). F2-isoprostanes have a vasoconstrictive effect. Cracowski et al., *Cardiovascular pharmacology and physiology of the isoprostanes, Fundamental & Clinical Pharmacology* 20(5): 417-427 (2006)(attached at Tab 2). Taken together, it should be expected that administration of CLA would result in an increase in blood pressure.

5. Second, administration of other agents known to be effective for weight loss can result in increased hypertension. Ephedrine, a commonly used, biologically active weight loss supplement is one such example. As established in Haller and Benowitz, *Adverse Cardiovascular and Central Nervous System Events Associated with Dietary Supplements Containing Ephedra Alkaloids*, *New England J. Med.* 343(25):1833-1838 (2000)(attached at Tab 3), ephedrine can cause an increase in hypertension.

6. The Examiner's argument that it would be obvious to use CLA to decrease hypertension because CLA administration also causes weight loss lacks scientific merit. How an agent such as CLA acts in the body is complex. Whether CLA causes an increase or decrease in hypertension, or has no effect at all, is determined by a variety of factors that have no relation to weight loss. It is not scientifically valid to draw a conclusion that because an agent causes weight loss, it can also be expected to decrease hypertension. The references cited by the Examiner contain no data that can be interpreted in this manner.

7. I further declare that all statement made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of title 18 of the United States

**PATENT**

U.S. Appln. Ser. No.: 09/410,484

Attorney Docket No. NATNUT-03972

Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.



Asgeir Sæbo

Date:

March 26th. 2007

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# *Fundamental & Clinical* Pharmacology

REVIEW  
ARTICLECardiovascular pharmacology and  
physiology of the isoprostanesJean-Luc Cracowski<sup>a\*</sup>, Thierry Durand<sup>b</sup><sup>a</sup>Laboratoire de Pharmacologie, Inserm ESPRI, HP2 EA 3745, Faculté de Médecine de Grenoble, France<sup>b</sup>UMR CNRS, 5074, Faculté de Pharmacie, Université Montpellier I, Montpellier, France

## Keywords

atherosclerosis,  
hypertension,  
ischemia,  
isoprostane,  
lipid peroxidation,  
vasoconstriction

## ABSTRACT

$F_2$ -isoprostanes are a complex family of compounds produced from arachidonic acid via a free radical-catalyzed mechanism. Their quantification as a pathophysiological biomarker provides a unique opportunity to investigate lipid peroxidation in vascular diseases. Their measurement also provides an interesting biomarker for the rational dose selection of antioxidants in vascular diseases where oxidative stress might be involved. In addition to their use as biomarkers, some isoprostanes possess a biological activity. The 15-series  $F_2$ - and  $E_2$ -isoprostanes mediate vasoconstriction in different vascular beds and species. In addition, 15- $F_2$ -IsoP induces smooth muscle cells mitogenesis and monocyte adhesion to endothelial cells. The data available supports but does not prove the hypothesis that isoprostanes are involved in vascular physiology and pathogenesis.

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## INTRODUCTION

Isoprostanes are a complex family of compounds produced from arachidonic acid via a free radical-catalyzed mechanism. In vitro generation of auto-oxidation products derived from polyunsaturated fatty acids was described more than 30 years ago [1,2]. However, the first demonstration that these compounds were produced in humans was shown in 1990 by Morrow et al. [3], who reported the discovery of prostaglandin- $F_2$ -like compounds, termed  $F_2$ -isoprostanes, generated by free radical-induced peroxidation of arachidonic acid. Since that time,  $F_2$ -isoprostanes have been used extensively as clinical markers of lipid peroxidation in cardiovascular disorders. These compounds are not only biomarkers. Indeed, the 15-series  $F_2$ - and  $E_2$ -isoprostanes possess a pharmacological activity on the blood vessels.

ISOPROSTANE STRUCTURE AND  
SYNTHESIS

Depending on which of the labile hydrogen atoms of arachidonic acid is first abstracted by free radicals, three

initial arachidonoyl radicals can be formed following free radical attack. These radicals form four prostaglandin- $H_2$ -like compounds that can then be fully reduced to form four prostaglandin  $F_{2x}$  regioisomers (Figure 1), or rearranged to form prostaglandin  $E_2$  and  $D_2$  regioisomers. Two mechanisms, based on the formation of a 'dioxetane' intermediate, via a 4-*exo*-cyclization or a  $\beta$ -fragmentation followed by successive 5-*exo*-cyclizations have been proposed recently for the formation of these compounds [4]. Because each  $F_2$ -isoprostane regioisomer comprises eight diastereoisomers, 64 different  $F_2$ -isoprostanes can be generated.

Isoprostanes were formerly named according to the prostaglandin  $F_{2x}$  chemical structure. They differ from prostaglandins by the *cis*-stereochemistry of the five-membered ring junction instead of the *trans*-stereochemistry of the prostaglandin  $F_{2x}$ . Because the first isoprostanes described were the 15-series, they were formerly named according to this major difference, the first isoprostane being named 8-*iso*-prostaglandin  $F_{2x}$  or 8-*epi*-prostaglandin  $F_{2x}$ . However, such a nomenclature does not allow the differentiation of the numerous isomeric structures. Two nomenclatures were proposed

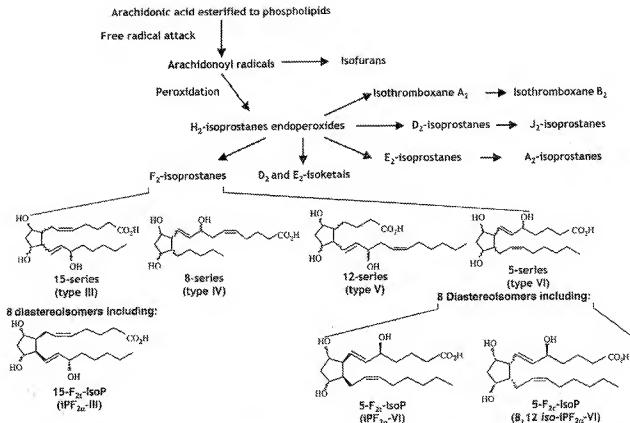


Figure 1 The isoprostane (IsoP) pathway. Free radical attack of arachidonic acid results in the formation of arachidonoyl radicals, which, following peroxidation, form four prostaglandin-H<sub>2</sub>-like compounds that can then be fully reduced to form four prostaglandin F<sub>2a</sub> regioisomers [those of the 15-series (type III), 8-series (type IV), 12-series (type V) and 5-series (type VI)], or rearranged to form prostaglandin H<sub>2</sub> and D<sub>2</sub> regioisomers. Each regioisomer comprises eight diastereoisomers and so 64 different F<sub>2</sub>-isoprostanes can be generated.

recently, both of which enable an easy differentiation of the isoprostane isomers. Taber et al. [5] nomenclature was filed with the Eicosanoid Nomenclature Committee, and approved by the International Union of Pure and Applied Chemistry. Rokach et al. [6] also proposed a nomenclature that enables the differentiation of the regioisomers. In 1997, this nomenclature was modified to be applicable to isoprostane-like compounds derived from eicosapentaenoic and docosahexaenoic acid. The concomitant use of these three different nomenclatures is confusing for the nonspecialist, and we propose that the old nomenclature (e.g. 8-iso-prostaglandin F<sub>2a</sub>) be definitively abandoned, and that Taber's nomenclature be encouraged. The different nomenclatures used to name the main isoprostanes are given in Figure 2.

Several *in vitro* studies have suggested a cyclooxygenase (COX)-dependent formation of 15-F<sub>2</sub>-IsoP [7–9]. An efficient *in vivo* production of 15-F<sub>2</sub>-IsoP through the COX pathway would reduce its accuracy as

a valid marker of lipid peroxidation. In contrast to the *in vitro* data, clinical studies clearly showed that COX inhibition was unable to decrease the formation of F<sub>2</sub>-isoprostanes in healthy subjects as well as patients, suggesting that F<sub>2</sub>-isoprostanes are formed via a non-COX-dependent mechanism *in vivo* [10–14]. Furthermore, in conditions of increased COX-2 expression following intravenous lipopolysaccharide challenge, the formation of 15-F<sub>2</sub>-IsoP and of 5-series isomers was not altered by COX inhibitors in healthy volunteers, whereas prostanoid production was decreased, further suggesting a COX-independent pathway of F<sub>2</sub>-isoprostane synthesis [15]. Finally, an *in vivo* COX-dependent formation of IPF<sub>2</sub>-III has been shown in the rat but not in humans [16]. Altogether, these data suggest that although a COX-dependent formation can be demonstrated *in vitro*, this does not occur *in vivo* in humans, meaning that 15-F<sub>2</sub>-IsoP as well as the 5-series isomers can be used as biomarkers of lipid peroxidation *in vivo*.

Chemical structure	Former nomenclature	Taber's nomenclature	Rokach's nomenclature
	8-iso-PGF <sub>2α</sub>	15-F <sub>2α</sub> -IsoP	IPF <sub>2α</sub> -III
	8-iso-PGE <sub>2</sub>	15-E <sub>2</sub> -IsoP	IPF <sub>2</sub> -III
	2,3-dinor-5,6-dihydro-8-iso-PGF <sub>2α</sub>	2,3-dinor-5,6-dihydro-15-F <sub>17</sub> -IsoP	2,3-dinor-5,6-dihydro-IPF <sub>2α</sub> -III
	Not available	5-F <sub>2α</sub> -IsoP	IPF <sub>2α</sub> -VI
	Not available	5-F <sub>2α</sub> -IsoP	8,12-iso-IPF <sub>2α</sub> -VI
	8-iso-PGF <sub>2α</sub>	15-F <sub>2α</sub> -IsoP	IPF <sub>2α</sub> -III

Figure 2 Nomenclature of the isoprostanes.

## ISOPROSTANE QUANTIFICATION

Quantification of F<sub>2</sub>-isoprostanes is used as a reliable marker of lipid peroxidation *in vivo* [17], and several methods are currently used [18] including gas chromatography (GC)-mass spectrometry (MS), which might be associated with an immunoaffinity extraction, GC-tandem MS, and liquid chromatography-tandem MS. These methods are reviewed in detail elsewhere [19]. They are specific but their cost and technology limit their routine use. Measurement of urinary 15-F<sub>2α</sub>-IsoP by radioimmunoassay has been validated and constitutes a valid and easier alternative to GC-MS [20]. Enzyme immunoassays have also been developed to measure levels of F<sub>2</sub>-isoprostanes but the antibodies used have not been tested for cross-reactivity with the numerous F<sub>2</sub>-isoprostane isomers and their metabolites. The results obtained using enzyme immunoassays sometimes differ from those obtained using GC-MS assays and therefore immunoassays should be considered as semi-quantitative indices of F<sub>2</sub>-isoprostanes.

## VASCULAR PHARMACOLOGY OF THE ISOPROSTANES

Isoprostanes are formed *in situ* on phospholipids, at sites of free radical generation. Once released from cell

membranes by phospholipases, isoprostanes circulate in the plasma in free forms and are therefore susceptible to activate membrane receptors. Most studies have focused on the biological activity of 15-F<sub>2α</sub>-IsoP, the first isoprostane commercially available. 15-F<sub>2α</sub>-IsoP is a vasoconstrictor in most species and vascular beds, both *in vitro* and *in vivo*, following intravenous administration (see [21,22] for full reviews). These constrictor properties are not specific to the blood vessels and have been demonstrated in the lymphatic vessels, the bronchi, the gastrointestinal tract and the uterus. In addition, it stimulates mitogenesis in uterine vascular smooth muscle cells [23]. The available data strongly suggest that the effects of 15-F<sub>2α</sub>-IsoP on blood vessels are mediated by the activation of the TP receptors (thromboxane A<sub>2</sub>/prostaglandin H<sub>2</sub> receptors), acting as a full or partial agonist [24,25], although some responses including mitogenesis appear to be at least in part TP receptor independent. The existence of a specific isoprostane receptor has been suggested but remains to be elucidated [26]. In addition, preliminary data suggest that isoprostanes, as well as their precursor arachidonic acid, are other lipid ligands for the peroxisome proliferator activated receptors (PPAR) [27].

The effects of 15-F<sub>2α</sub>-IsoP on platelets are complex. When incubated with subthreshold concentrations of ADP, thrombin, collagen and arachidonic acid, 15-F<sub>2α</sub>-IsoP causes irreversible platelet aggregation, dependent

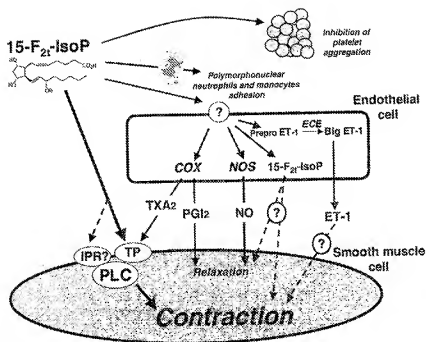


Figure 3 Schematic representation of the pharmacological activities of the isoprostane 15-F<sub>2t</sub>-Isop on the interface blood vessels. COX, cyclo-oxygenase; TXA<sub>2</sub>, thromboxane A<sub>2</sub>; PGI<sub>2</sub>, prostacyclin; NO, nitric oxide; TP, prostaglandin H<sub>2</sub>/thromboxane receptor; IPR, specific isoprostane receptor; PLC, phospholipase C; ET-1, endothelin-1; ECE, endothelin conversion enzyme. For a better comprehension, the thickness of the arrows are correlated to the scientific evidence of these mechanisms. Such mechanisms are likely to differ within species, as well as within the vessel types.

on thromboxane generation, while 15-F<sub>2t</sub>-Isop alone induces weak, reversible aggregation, only at high concentrations [28]. As 15-F<sub>2t</sub>-Isop is a partial agonist at the prostanoid TP receptor on platelets, it might inhibit the pro-aggregatory effects of TP receptor stimulation. Indeed, in human whole blood, 15-F<sub>2t</sub>-Isop is anti-aggregatory [29]. Several authors suggested that increased isoprostane formation is one of the factors involved in aspirin resistance, but a full demonstration of this hypothesis is not available to date [30–33].

15-F<sub>2t</sub>-Isop-induced contraction is modulated by the endothelium through the release of NO, i.e. endothelium removal increases 15-F<sub>2t</sub>-Isop contraction [21]. In addition, 15-F<sub>2t</sub>-Isop induces both thromboxane A<sub>2</sub> and endothelin-1 release from endothelial cells (Figure 3). In comparison with the huge data available for 15-F<sub>2t</sub>-Isop, few are available for other isomers. Nevertheless, other isoprostanes belonging to the 15 series of the F-family isoprostanes, such as 9-*epi*-15-F<sub>2t</sub>-Isop and 15-*epi*-15-F<sub>2t</sub>-Isop are biologically active, although less potent than 15-F<sub>2t</sub>-Isop [21]. The 5-series and 15-series F<sub>2</sub>-isoprostanes are produced in approximately equal amounts *in vivo* whereas the 8-series and 12-series F<sub>2</sub>-isoprostanes are produced in lower amounts [34]. In human urine and plasma, the 5-series (e.g. 5-F<sub>2t</sub>-Isop and 5-F<sub>2c</sub>-Isop) was found to be the most abundant F<sub>2</sub>-isoprostanes [35]. Both the 15-series and the 5-series are easily detectable in human urine and plasma. However, in contrast to the

15-series F<sub>2</sub>-isoprostanes, the 5-series F<sub>2</sub>-isoprostanes have no vasomotor effect in different species and vascular beds [36] and as such are unlikely to be involved in the pathogenesis of vascular diseases. In addition to the vasoconstrictor and mitogenic effects, 15-F<sub>2t</sub>-Isop and 15-E<sub>2t</sub>-Isop induce monocyte adhesion to endothelial cells [37,38], whereas 15-F<sub>2t</sub>-Isop is a specific activator of rapid neutrophil adhesion [39]. Furthermore, 15-F<sub>2t</sub>-Isop induces cerebral endothelial cell death [40]. Endothelial cells are one of the first targets of oxidative stress in atherogenesis and ischemia-reperfusion injury. Whether isoprostanes may be one of pathogenic mediators remains to be tested.

Compared with the F<sub>2</sub>-isoprostanes, E<sub>2</sub>-isoprostanes are more potent *in vitro*. 15-E<sub>2t</sub>-Isop is more potent than 15-F<sub>2t</sub>-Isop in systemic and pulmonary vessels, its contraction being mediated through TP receptor, and EP<sub>3</sub> receptor activation in the pulmonary vasculature [41,42]. In addition, 15-E<sub>2t</sub>-Isop may induce a relaxation through EP receptors [43]. However, because no data are available concerning the production of E<sub>2</sub>-isoprostanes in cardiovascular disorders, it is premature to conclude concerning the potential role of such compounds in cardiovascular pathogenesis.

One should keep in mind that Morrow et al. showed [44] that A<sub>2</sub>-isothromboxanes are formed *in vivo*. Due to the inherent instability of the thromboxane A<sub>2</sub> ring, no data concerning the vascular effects of A<sub>2</sub>-isothromb-



oxanes are available. However, regarding the potency of thromboxane  $A_2$ -induced contractions, studies using stable  $A_2$ -isothromboxanes analogues are awaited with interest.

To date, the metabolism of 15- $F_{2t}$ -IsoP leads to two major metabolites in humans: 2,3-dinor-15- $F_{2t}$ -IsoP and 2,3-dinor-5,6-dihydro-15- $F_{2t}$ -IsoP [45,46]. The metabolite 2,3,4,5-tetranor-13,14-dihydro-15-keto-15- $F_{2t}$ -IsoP [47] was identified as the major metabolite in rabbits but there is no evidence that such a metabolite is produced in humans. Although most prostanoid metabolites are biologically inactive, surprisingly, a recent report showed that 2,3-dinor-5,6-dihydro-15- $F_{2t}$ -IsoP exhibits contraction that is comparable with that of 15- $F_{2t}$ -IsoP in porcine brain microvessels [48]. By contrast, unlike 15- $F_{2t}$ -IsoP, neither 2,3-dinor-5,6-dihydro-15- $F_{2t}$ -IsoP nor 2,3-dinor-15- $F_{2t}$ -IsoP had any constrictor or dilator effects on the rat thoracic aorta [49]. Such disparate observations need to be further investigated in different species and vascular beds.

Recently, isoprostane-like compounds derived from eicosapentaenoic and docosahexaenoic acids have been discovered *in vivo* [50–52]. Among the  $F_3$ -isoprostanes formed, 15- $F_{3t}$ -IsoP possesses either no biological effect or might induce a weak relaxation in human airways. Preliminary data suggest that  $F_4$ -neuroprostanes possess no vascular effects.

## ISOPROSTANES AS A BIOMARKER OF LIPID PEROXIDATION IN VASCULAR DISEASES

Isoprostanes have been measured in biological fluids such as urine, plasma, exhaled breath condensate, bronchoalveolar lavage fluid, bile, cerebrospinal, seminal and pericardial fluids. They are also detectable in normal tissues, including umbilical cords [53]. The main advantage of urinary measurements is that both 15- $F_{2t}$ -IsoP and 5- $F_{2t}$ -IsoP are not formed *ex vivo* by auto-oxidation in urine, unlike in plasma samples.

Cigarette smoking was one of the first conditions in which an increase in  $F_2$ -isoprostane levels was demonstrated [54]. This increase is reduced after 2 weeks of abstinence from smoking [14,54] and almost reaches the values of nonsmokers 4 weeks after quitting smoking [55]. Short-term cigarette smoking increases exhaled breath condensate  $F_2$ -isoprostane concentrations [56], but not plasma levels of  $F_2$ -isoprostane [54]. Restarting smoking after quitting and passive smoking are associated with an increase in plasma levels of  $F_2$ -isoprostane

[57,58]. Interestingly, 15- $F_{2t}$ -IsoP concentrations were approximately twice as high in umbilical cords from newborn babies of smoking mothers compared with those of nonsmoking mothers [53]. Together, these data provide evidence that cigarette smoking is associated with a chronic increased lipid peroxidation *in vivo*.

The measurement of isoprostanes in biological fluids has prompted clinical investigations on the pathophysiological role of lipid peroxidation in cardiovascular diseases (Table 1). Among the biological fluids available, most studies were performed on urine because of the non-invasiveness of the procedure and the lack of artifactual generation. A strong link between lipid peroxidation and vascular diseases associated with ischemia–reperfusion, atherosclerosis and inflammation has been suggested by the elevated levels of lipid peroxidation observed in such diseases.

In addition to being a pathophysiological marker, the quantification of  $F_2$ -isoprostanes might represent a prognostic marker. Indeed, Schvedhelm *et al.* [59] showed in a case–control study that urinary 15- $F_{2t}$ -IsoP level was a strong independent concentration-dependent risk marker of coronary heart disease. In addition, there is a relationship between plasma  $F_2$ -isoprostanes and early development of coronary artery calcifications [60]. There are currently no published clinical studies aimed at testing isoprostanes as a long-term prognostic marker, with strong endpoints such as mortality or morbidity, but cohort studies are on-going.

## ISOPROSTANES: EMERGING ROLE IN VASCULAR PHYSIOLOGY AND DISEASE?

An important issue to resolve is whether the same effects observed *in vitro* are observed consistently *in vivo* at physiological concentrations and whether these effects contribute to pathological states *in vivo*. Basal plasma concentrations of 15- $F_{2t}$ -IsoP have been found to range from approximately  $10^{-10}$  to  $5 \times 10^{-10}$  mol  $L^{-1}$  in plasma samples. These concentrations are unlikely to induce a systemic vasoactive effect considering the  $EC_{50}$  values of 15- $F_{2t}$ -IsoP observed *in vitro* [21,61]. However,  $F_2$ -isoprostanes are released at the site of free radical injury and then diluted in the circulation and therefore local concentrations might be sufficiently high to induce regional vasoconstriction. The concentrations of 15- $F_{2t}$ -IsoP and 5- $F_{2t}$ -IsoP are increased markedly in the coronary sinus following coronary angioplasty [62]. However, 15- $F_{2t}$ -IsoP concentrations are in the

Table 1  $E_2$ -isoprostane quantification in human cardiovascular diseases.

Disease	Isoprostanes quantified	Tissue or biological fluid tested	Method	Results in comparison with a control group	References
Essential hypertension	$15-F_2t\text{-isoP}$	Urine	EIA and GC-MS	NSD	[86-88]
Hypertensive patients with renovascular disease	$15-F_2t\text{-isoP}$	Urine	EIA	Increased	[86]
Heart failure	$15-F_2t\text{-isoP}$	Urine, pericardial fluid	LC-MS-MS and EIA	Increased	[89-92]
Atherosclerosis	$15-F_2t\text{-isoP}$ and $5-F_2t\text{-isoP}$	Atherosclerotic lesions from carotid endarterectomy	GC-MS and RIA	Increased	[93-95]
Ruptured abdominal aortic aneurysm	$15-F_2t\text{-isoP}$	Plasma	EIA	Increased	[96]
Stable coronary heart disease	$15-F_2t\text{-isoP}$	Urine	GC-MS and RIA	NSD	[97,98]
Unstable angina	$15-F_2t\text{-isoP}$	Urine	RIA	Increased	[98]
Reperfusion following myocardial infarction and cardiopulmonary bypass	$15-F_2t\text{-isoP}$ and $5-F_2t\text{-isoP}$	Urine	GC-MS	Increased	[97,98]
Coronary angioplasty	$15-F_2t\text{-isoP}$ and $5-F_2t\text{-isoP}$	Urine and coronary sinus	GC-MS	Increased	[92,99]
Systemic sclerosis (scleroderma)	metabolites	Urine	GC-MS AND EIA	Increased	[100-104]
Antiphospholipid antibodies syndrome	$15-F_2t\text{-isoP}$ and $5-F_2t\text{-isoP}$	Urine	GC-MS and EIA	Increased	[105,106]
Raynaud's phenomenon	$15-F_2t\text{-isoP}$	Urine	GC-MS	NSD	[101,104]
Pulmonary hypertension	$15-F_2t\text{-isoP}$	Urine	GC-MS	Increased	[107]
Acute ischemic stroke	$15-F_2t\text{-isoP}$	Urine	RIA	No variation over 72 h	[108]
Migraine	$15-F_2t\text{-isoP}$	Urine	RIA	NSD	[109]
Preeclampsia	$15-F_2t\text{-isoP}$ and $5-F_2t\text{-isoP}$	Plasma, urine and saliva and placental tissue	GC-MS and EIA	Conflicting results among studies	[110-116]

EIA, enzyme immunoassay; GC-MS, gas chromatography-mass spectrometry; LC-MS-MS, liquid chromatography-mass spectrometry; NSD, not significantly different; RIA, radioimmunoassay.

nanomolar range, and thus unlikely to contribute to epicardial coronary artery vasoconstriction [61,63].

No specific inhibition of 15-F<sub>2t</sub>-IsoP or other isoprostanes vascular effects can currently be achieved. However, TP receptor antagonists but not aspirin are effective in atherosclerosis inhibition in apo E knock-out mice, showing that TP receptors blockade by S18886 is effective by a mechanism independent of platelet-derived thromboxane A<sub>2</sub> [64], whereas isoprostanes suppression with vitamin E retards atherogenesis in the same animal model [65]. Similarly, TP receptor antagonism by L670596, but not COX-2 inhibition prevented pulmonary hypertension and endothelin-1 upregulation in 60% O<sub>2</sub>-mediated pulmonary hypertension in newborn rats [66]. In addition to these animal data, a recent study showed that in patients suffering from coronary artery disease, S18886, a TP receptor antagonist improved acetylcholine-induced and flow-mediated vasodilation in patients treated with aspirin [67]. An hypothesis is that endogenous TP receptor activation induced by 15-F<sub>2t</sub>-IsoP or other isoprostanes may be involved in the COX-independent effects of TP receptors antagonists [68]. However, because TP receptors share other endogenous ligands such as prostaglandin H<sub>2</sub> or hydroxyecosatrienoic acids (HETEs), such data give strength to the hypothesis that isoprostanes are involved in the vascular physiology and pathogenesis, but does not enable a definitive conclusion. Given that many isomeric isoprostanes exist, one should not focus only on 15-F<sub>2t</sub>-IsoP. Other 15-series F<sub>2</sub>-isoprostanes are biologically active, as well as F<sub>2</sub>-isoprostanes [22] and large field of investigations are still unexplored.

### ISOPROSTANES AS A PHARMACOLOGICAL TOOL FOR DRUG EVALUATION

In the past decade, most attention has focused on the effect of an antioxidant therapeutic strategy, including the use of vitamin E, in cardiovascular and nephrological diseases, with mixed results. Measurement of F<sub>2</sub>-isoprostanes currently represents a valuable pharmacological tool for the evaluation of antioxidant therapy, and should be used in the rational selection of antioxidant dosages. Vitamin E supplementation induced a reduction of urinary 15-F<sub>2t</sub>-IsoP levels in patients with cystic fibrosis [69], type 2 diabetes [11] and homozygous homocystinuria [70], but not in patients with systemic sclerosis [71]. In addition, such a reduction was dose dependent in hypercholesterolemic patients [10]. The

vitamin E-dependent reduction in the concentrations of F<sub>2</sub>-isoprostane was observed in clinical conditions of enhanced oxidative stress. By contrast, supplementation with vitamin E had no effects on F<sub>2</sub>-isoprostane levels in either moderate cigarette smokers [72], or healthy adults [73]. In the latter studies, the supplementation was associated with a significant dose-dependent increase in circulating concentrations of vitamin E. In addition, vitamin E supplementation in cigarette smokers on a high polyunsaturated fatty acid diet caused an increase in the plasma levels of F<sub>2</sub>-isoprostane [74]. Together, these studies suggest that vitamin E supplementation has antioxidant effects in patient populations that are characterized by high rates of lipid peroxidation. Patrignani et al. [72] hypothesized that the basal rate of lipid peroxidation may be an important determinant of the response to vitamin E supplementation, and could explain the variable effects of vitamin E supplementation in large clinical trials. Several studies favor such a hypothesis. Oral treatment with raxofelast, a vitamin-E-like antioxidant, induced a significant reduction of plasma concentrations of 15-F<sub>2t</sub>-IsoP in type 2 diabetes but had no effect in healthy subjects [75]. In addition, vitamin C supplementation reduced urinary 15-F<sub>2t</sub>-IsoP levels in patients with stroke [76], and chronic alcoholic liver disease but not in patients with hepatitis C cirrhosis, in whom endogenous vitamin C and E concentrations did not significantly differ compared with controls [77]. Furthermore, vitamin C did not decrease urinary F<sub>2</sub>-isoprostane and metabolite levels in healthy young women [78], and in young subjects with a limited history of cigarette smoking [79]. Together, these data strengthen the need to incorporate the measurement of surrogate end-points such as F<sub>2</sub>-isoprostanes in large-scale antioxidant clinical trials.

In addition to drug evaluation, F<sub>2</sub>-isoprostane measurement could be used to test the antioxidant properties of the diet. F<sub>2</sub>-isoprostane levels were decreased following supplementation with eicosapentaenoic acid or docosahexaenoic acid [80], fish meals in diabetic patients [81], olive oil [82], soy-containing isoflavone [83], gazpacho [84], and flavanol-rich cocoa [85].

### CONCLUSION

Isoprostanes are a complex family of compounds produced from arachidonic acid via a free radical-catalyzed mechanism. Some isoprostanes induce vasoconstriction, mitogenesis and monocyte adhesion. The quantification of F<sub>2</sub>-isoprostanes as a pathophysiological biomarker

provides a unique opportunity to investigate lipid peroxidation in vascular diseases. Their measurement provides an interesting biomarker for the rational dose selection of antioxidants in vascular diseases where oxidative stress might be involved. The data available supports but does not prove the hypothesis that isoprostanes are involved in vascular physiology and pathogenesis.

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# Arteriosclerosis, Thrombosis, and Vascular Biology

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## Brief Reviews

### Diet and Murine Atherosclerosis

Godfrey S. Getz, Catherine A. Reardon

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### Smoking, Metalloproteinases, and Vascular Disease

Todd S. Perlestein, Richard T. Lee

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### Influence of Cardiovascular Risk Factors on Endothelial Progenitor Cells: Limitations for Therapy?—ATVB In Focus

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Ton J. Rabelink, Thomas F. Luscher

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## Vascular Biology

### Importance of Junctional Adhesion Molecule-A for Neointimal Lesion Formation and Infiltration in Atherosclerosis-Prone Mice

Alma Zernecke, Elisa A. Liehn ... Christian Weber

Web Site Feature

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#### Abstract

Junctional adhesion molecule-A (JAM-A) has recently been implicated in leukocyte recruitment on early atherosclerotic endothelium. Our data provide the first evidence to our knowledge for a crucial role of JAM-A in accelerated lesion formation and monocyte infiltration in atherosclerosis-prone mice. 272

### Aspirin Has A Gender-Dependent Impact on Antiinflammatory 15-Epi-Lipoxin A<sub>4</sub> Formation: A Randomized Human Trial

Nan Chiang, Shelley Hurwitz ... Charles N. Serhan

Web Site Feature

e14-e17

#### Abstract

In a double-blinded clinical trial, low-dose aspirin significantly increased the antiinflammatory mediator, aspirin-triggered-15-epi-lipoxin A<sub>4</sub>. A post-trial analysis showed that the age trends in aspirin-triggered-15-epi-lipoxin A<sub>4</sub> formation are significantly different with genders: a positive correlation in women and a negative correlation in men. Therefore, aspirin-triggered-15-epi-lipoxin A<sub>4</sub> may be related to the gender-specific clinical impact of aspirin. 273

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# Conjugated Linoleic Acid Impairs Endothelial Function

Justin S.W. Taylor, Simon R.P. Williams, Rhian Rhys, Phillip James, Michael P. Frenneaux

**Objectives**—To determine the effect of dietary supplementation with conjugated linoleic acid (CLA) on body mass index (BMI), body fat distribution, endothelial function, and markers of cardiovascular risk.

**Methods and Results**—Forty healthy volunteers with BMI  $>27$  kg/m<sup>2</sup> were randomized to receive a CLA isomeric mixture or olive oil in a 12-week double-blind study. Subcutaneous body fat and abdominal/hepatic fat content were assessed using skin-fold thicknesses and computed tomography scanning, respectively. Endothelial function was assessed by brachial artery flow-mediated dilatation (FMD). Plasma isoprostanes were measured as an index of oxidative stress. CLA supplementation did not result in a significant change in BMI index or total body fat. There was a significant decrease in limb ( $-7.8$  mm,  $P<0.001$ ), but not torso skin-fold thicknesses or abdominal or liver fat content. Brachial artery FMD declined ( $-1.3\%$ ,  $P=0.013$ ), and plasma F2-isoprostanes increased ( $+91$  pg/mL,  $P=0.042$ ).

**Conclusions**—A CLA isomeric mixture had at most modest effects on adiposity and worsened endothelial function. On the basis of these results, the use of the isomeric mixture of CLA as an aid to weight loss cannot be recommended. (*Arterioscler Thromb Vasc Biol.* 2006;26:307-312.)

**Key Words:** body composition ■ conjugated linoleic acid ■ endothelial function ■ obesity ■ oxidative stress

Abdominal obesity<sup>1</sup> and the associated dysmetabolic syndrome<sup>2</sup> confer increased cardiovascular risk. Dietary modification with n-3 polyunsaturated fatty acids appears to reduce the risk of coronary artery disease and improve mortality.<sup>3-5</sup> Conjugated linoleic acid (CLA) is a naturally occurring fatty acid and is found in dairy products and meat from ruminants. It differs from the better known linoleic acid by having an extra carbon-carbon double bond. It has been widely promoted in the lay press,<sup>6</sup> with claims that it can prevent and treat cancer,<sup>7</sup> prevent heart disease,<sup>8</sup> improve immune function,<sup>9</sup> and treat obesity.<sup>10</sup> Whereas some of these effects are supported by studies in animals,<sup>9,11</sup> there is conflicting published human research on CLA, and in particular there have been no conclusive studies measuring its effect on markers of cardiovascular risk. Furthermore, it is now realized that the different isomers of CLA may have very different biological properties and may have different mechanisms of action.<sup>12</sup> The 2 main CLA isomers that have been studied are 9,11 and 10,12 CLA. Other studies have suggested that CLA supplementation may increase oxidative stress, although this was not proven.<sup>16,17</sup> Furthermore, the small number of studies thus far published have suggested that in humans, weight loss produced by CLA supplementation is at most modest.<sup>10,13,16-18</sup> To assess the efficacy of CLA as an aid to weight loss and its effect on cardiovascular risk factors, we undertook a double blind study examining the effects of a commercially available isomeric mixture of CLA on body weight, body fat mass and distribution, endothelial

function, insulin sensitivity, and markers of oxidative stress in overweight middle-aged men.

## Methods

### Subjects

Forty nonsmoking white men, aged 35 to 60, without diabetes, hypertension, or cardiovascular disease, with a body mass index (BMI)  $>27$  kg/m<sup>2</sup> were recruited from the local community through media advertisements. All subjects gave informed written consent and the protocol was approved by the local research and ethics committee.

### Protocol

Subjects were randomly assigned to receive 4.5 g/d of CLA (isomeric mixture 60 calories/d) or olive oil (54 calories/d). The randomization was performed by an independent observer who also matched subjects by age and BMI. The isomeric mixture contained 35% 9c,11t CLA, 36% 10c,12 CLA, 1% to 2% 9c,11c and 10c,12c CLA, 1.5% 9c,11t and 10c,11t CLA, and  $<1\%$  8c,10 and c,11,13 CLA. The CLA and olive oil capsules were supplied by Natural Lipids (Hovdebygd, Norway). All vascular measurements were made in the morning after an overnight fast, at the beginning of the study, and after 12 weeks of supplementation.

### Body Composition

Body weight and height were measured and BMI calculated. Skin-fold thicknesses were measured according to standard guidelines<sup>19</sup> with Harpenden skinfold calipers (Holtain Ltd, Crymch, UK) at the following sites: biceps, triceps, front mid-thigh, medial calf, subscapular, mid-axillary, and abdominal. Abdominal, waist, and hip girths were measured. All measurements were made in triplicate and averaged. Bioelectrical impedance analysis was performed using the

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tetrapolar method and a Bodystat 1500 analyser (Bodystat Ltd, Isle of Man). Abdominal adipose tissue and liver fat were measured using two computed tomography (CT) images as described previously.<sup>20</sup> Abdominal adipose tissue is presented as a surface area at the level of the fourth lumbar vertebrae, and hepatic and splenic fat is presented as radiographic density in Hounsfield units (HU). Images were acquired using a Somatom Plus 4 scanner. CT slices were 10 mm in thickness and were obtained at 120 kV and 200 mA, with a 42-cm field of view and a 512×512 matrix. Image analysis was performed using dedicated software (SliceOmatic Version 4.2; Tomovision, Montreal, Canada).

### Endothelial Function

Changes in brachial artery diameter in response to reactive hyperemia (FMD) were measured noninvasively using a high-resolution ultrasonic wall-tracking system (Vadirec Wall-track System™) as previously validated.<sup>21,46,50</sup> Studies were performed at a controlled temperature of 21°C, with subjects supine and their arm held outstretched on a cushion. Baseline measurements of internal brachial artery diameter were taken after 15 minutes of rest. Reactive hyperemia was produced by releasing a pediatric sphygmomanometer wrist cuff inflated to systolic pressure plus 50 mm Hg for 5 minutes. Internal brachial artery diameter was measured every minute after cuff release, and the maximum change from baseline was used to calculate FMD. Data are presented as the percentage diameter change from baseline in the brachial artery.

### Laboratory Measurements

Venous blood samples were freshly analyzed for glucose, insulin, cholesterol (including low-density lipoprotein and high-density lipoprotein), and C-reactive protein. Further samples were centrifuged and the supernatant frozen at -80°C. These samples were analyzed later using enzyme immuno-linked assays for leptin (Alexis), adiponectin (Biogenesis UK), F2-isoprostanes (Alexis), and tumor necrosis factor- $\alpha$  (paired antibody enzyme-linked immunosorbent assay). Insulin sensitivity was measured indirectly using HOMA-IR [fasting serum insulin ( $\mu$ U/mL)×fasting plasma glucose (mmol/L)/22.5].

### Statistical Analysis

Analysis was performed using SPSS v11.5. Baseline values are presented as mean±SD. Variables with a skewed distribution were logarithmically transformed to achieve a normal distribution prior to analysis. Baseline comparison between the CLA and olive oil groups was assessed by Student's *t* test. The effects of treatment with CLA versus olive oil were assessed by using ANCOVA using baseline values as covariates and are presented with 95% confidence intervals. To avoid type I errors, post-hoc Bonferroni corrections were applied to the groups of primary objective measurements. Therefore, 2-tailed  $P<0.025$ ,  $P<0.005$ ,  $P<0.001$ ,  $P<0.025$ , and  $P<0.008$  were regarded as significant for blood pressure, skin-folds, girths, FMD, and abdominal CT measurements, respectively. A 2-tailed  $P<0.05$  was regarded as significant for primary objective measurements weight, and bioimpedance, and the exploratory blood analyses.

### Results

Baseline measurements did not differ significantly in the olive oil (19 subjects) and CLA (21 subjects) groups (Table 1). All subjects completed the study. The effect of 12 weeks of supplementation is shown in Table 2. The important changes are also displayed in the Figure. There were no significant changes in parameters in the olive oil group except for a decline in tumor necrosis factor- $\alpha$  ( $-61$  pg/mL [95% CI,  $-3$  to  $-120$ ];  $P=0.04$ ). In the CLA group there was no significant change in body mass ( $-1.1$  kg [95% CI,  $-2.3$  to  $0.04$ ];  $P=0.06$ ), BMI ( $-0.4$  kg/m<sup>2</sup> [95% CI,  $-0.8$  to  $0.03$ ];  $P=0.07$ ), or total body fat ( $-1$  % [95% CI,  $-2.5$  to  $0.5$ ];

TABLE 1. Characteristics of Olive Oil and CLA Groups at the Start of the Study

Baseline Characteristics	Olive Oil (n=19)	CLA (n=21)	P
Age, y	47±8	46±8	NS
Mass, kg	97±13	101±9	NS
BMI	39±3	33±3	NS
Fat, % (Bioimpedance)	29±3	26±4	NS
Systolic BP, mm Hg	128±13	122±10	NS
Diastolic BP, mm Hg	85±7	80±8	NS
Anthropometric measurements			
Skin-folds: Biceps, mm	12±4	11±4	NS
Skin-folds: Triceps, mm	20±5	19±5	NS
Skin-folds: Mid-thigh, mm	23±7	25±0	NS
Skin-folds: Medial calf, mm	16±4	16±5	NS
Skin-folds: Subscapular, mm	32±6	32±7	NS
Skin-folds: Mid-axillary, mm	23±4	24±6	NS
Skin-folds: Abdominal, mm	33±7	35±6	NS
Skin-folds: Limb, mm	72±17	72±18	NS
Skin-folds: Torso, mm	123±13	128±16	NS
Skin-folds: Torso:Limb	1.8±0.4	1.9±0.3	NS
Girth: Abdomen, cm	107±7	107±6	NS
Girth: Waist, cm	112±7	112±7	NS
Girth: Hip, cm	110±6	110±5	NS
Girth: Waist:Hip	1.02±0.03	1.01±0.04	NS
Girth: Abdomen:Hip	0.98±0.05	0.97±0.05	NS
Endothelial function			
Brachial artery, mm	3.8±0.5	3.7±0.4	NS
Brachial artery FMD, %	2.1±2.1	2.2±2.7	NS
Abdominal CT measurements			
Liver, HU	80±13	66±10	NS
Spleen, HU	64±2	61±2	NS
Liver:Spleen	0.93±0.18	1.08±0.18	NS
Visceral area, cm <sup>2</sup>	242±62	204±70	NS
Subcutaneous fat area, cm <sup>2</sup>	341±80	357±93	NS
Visceral:Subcutaneous fat	0.75±0.38	0.63±0.37	NS
Blood analyses			
LDL cholesterol, mmol/L	3.6±0.9	3.4±0.8	NS
HDL cholesterol, mmol/L	1.0±0.3	1.1±0.3	NS
Total cholesterol, mmol/L	5.6±0.9	5.4±0.8	NS
Triglycerides, mmol/L	2.3±1.3	1.8±1.0	NS
CRP, mg/L	5.2±2.9	4.8±2.7	NS
Glucose, mmol/L	5.2±0.6	5.1±0.7	NS
Insulin, mU/L	18±22	13±7	NS
HOMA-IR	4.6±6.4	3.1±1.8	NS
Leptin, ng/mL	21±12	22±16	NS
Adiponectin, pg/mL	8±6	9±6	NS
F2-isoprostanes, pg/mL	135±67	99±103	NS
Tumor necrosis factor- $\alpha$ , pg/mL	122±97	109±145	0.04

NS indicates not significant.

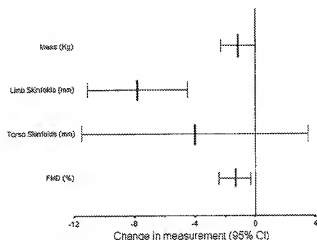
$P=0.18$ ). There was a significant decrease in limb ( $-7.8$  mm [95% CI,  $-11.1$  to  $-4.5$ ];  $P<0.001$ ), but not torso ( $-4.0$  mm [95% CI,  $-11.5$  to  $3.5$ ];  $P=0.29$ ) skin folds, and a significant increase in the total torso-to-limb skin-fold ratio ( $+0.13$  [95%

TABLE 2. Change in Measurements in Each Treatment Group After 12 Weeks of Supplementation Plus ANCOVA Analyses Showing the Overall Effect of Supplementation With CLA

	Olive Oil	CLA	Change in Measurement (ANCOVA) [95%CI]
Mass, kg	0.9±1.4	-0.2±1.9	-1.1 [-2.3 to 0.94] ( <i>P</i> =0.06)
BMI	0.3±0.5	-0.1±0.7	-0.4 [-0.8 to 0.05] ( <i>P</i> =0.07)
Fat, % (Bioimpedance)	1.1±2.9	0.1±1.7	-1 [-2.5 to 0.5] ( <i>P</i> =0.18)
Systolic BP, mm Hg	0.2±6.7	-0.4±9.1	-2.2 [-7.1 to 2.7] ( <i>P</i> =0.57)
Diastolic BP, mm Hg	-0.8±7.5	0.1±5.7	-1.3 [-5.4 to 2.8] ( <i>P</i> =0.52)
<b>Anthropometric measurements</b>			
Skin-folds: Biceps, mm	0.1±2.3	-1.4±1.5	-1.6 [-2.7 to -0.5] ( <i>P</i> =0.006)
Skin-folds: Triceps, mm	0.3±1.9	-1.1±2.4	-1.5 [-2.9 to 0.09] ( <i>P</i> =0.046)
Skin-folds: Mid-thigh, mm	0.4±2.1	-2.6±2.6	-2.8 [-4.3 to -1.3] ( <i>P</i> <0.001)
Skin-folds: Medial calf, mm	0.2±1.4	-1.5±1.5	-1.6 [-2.7 to -0.8] ( <i>P</i> =0.001)
Skin-folds: Sub-scapular, mm	0.9±3.3	-0.3±4.8	-1.3 [-3.8 to 1.2] ( <i>P</i> =0.29)
Skin-folds: Mid-axillary, mm	-0.4±2.8	-3.0±3.0	-2.3 [-4.0 to -0.6] ( <i>P</i> =0.011)
Skin-folds: Abdominal, mm	2.0±6.8	2.2±3.4	+1.4 [-1.8 to 4.3] ( <i>P</i> =0.36)
Skin-folds: Limb, mm	1.1±3.5	-6.6±6.0	-7.8 [-11.1 to -4.5] ( <i>P</i> <0.001)
Skin-folds: Torso, mm	2.9±13.5	-2.1±9.9	-4.0 [-11.5 to 3.5] ( <i>P</i> =0.29)
Skin-folds: Torso:Limb	0.02±0.18	0.15±0.15	+0.13 [0.03 to 0.24] ( <i>P</i> =0.017)
Girths: Abdomen, cm	0.1±1.3	-0.1±1.9	-0.2 [-1.4 to 0.9] ( <i>P</i> =0.69)
Girths: waist, cm	-0.3±2.1	0.5±2.5	+0.8 [-0.8 to 2.4] ( <i>P</i> =0.30)
Girths: hip, cm	0.4±1.4	-0.01±1.5	-0.4 [-1.3 to 0.6] ( <i>P</i> =0.44)
Girths: Waist:Hip	-0.003±0.03	0.01±0.22	+0.01 [-0.01 to 0.02] ( <i>P</i> =0.44)
Girths: Abdominal:hip	-0.01±0.03	-0.01±0.01	+0.01 [-0.01 to 0.02] ( <i>P</i> =0.42)
<b>Endothelial function</b>			
Brachial artery, mm	-0.17±0.60	-0.03±0.62	+0.06 [-0.29 to 0.41] ( <i>P</i> =0.72)
Brachial artery FMD, %	0.1±2.3	-1.3±2.5	-1.3 [-2.4 to -0.3] ( <i>P</i> =0.013)
<b>Abdominal CT measurements</b>			
Liver, HU	-0.7±5.7	-4.1±10.2	-3.9 [-9.9 to 2.2] ( <i>P</i> =0.20)
Spleen, HU	-2.2±4.8	0.7±2.8	1.1 [-1.7 to 3.8] ( <i>P</i> =0.44)
Liver:Spleen	0.01±0.11	-0.08±0.17	-0.09 [-0.21 to 0.03] ( <i>P</i> =0.12)
Visceral area, cm <sup>2</sup>	11.1±34.5	3.7±55.0	-4.5 [-33.6 to 24.6] ( <i>P</i> =0.76)
Subcutaneous fat area, cm <sup>2</sup>	-9.8±32.6	3.43±40.0	-12.6 [-39.5 to 14.2] ( <i>P</i> =0.34)
Visceral:Subcutaneous fat	-0.09±0.26	0.03±0.29	0.08 [-0.11 to 0.26] ( <i>P</i> =0.41)
<b>Blood analyses</b>			
LDL cholesterol, mmol/L	-0.2±0.5	-0.01±0.4	+0.1 [-0.1 to 0.3] ( <i>P</i> =0.41)
HDL cholesterol, mmol/L	-0.03±0.11	-0.09±0.13	-0.03 [-0.10 to 0.05] ( <i>P</i> =0.46)
Total cholesterol, mmol/L	-0.09±0.50	-0.07±0.44	-0.07 [-0.34 to 0.20] ( <i>P</i> =0.62)
Triglycerides, mmol/L	0.1±0.8	0.1±0.6	-0.1 [-0.6 to 0.4] ( <i>P</i> =0.57)
CRP, mg/L	-0.8±2.9	0.4±4.8	+0.9 [-1.4 to 3.2] ( <i>P</i> =0.41)
Glucose, mmol/L	0.3±0.8	0.2±0.8	-0.1 [-0.5 to 0.3] ( <i>P</i> =0.51)
Insulin, mIU/L	0.7±22.2	2.3±12.5	-3 [-11 to 6] ( <i>P</i> =0.50)
HOMA-IR	0.2±6.7	0.8±3.2	-0.8 [-3.2 to 1.6] ( <i>P</i> =0.50)
Leptin, ng/mL	1.3±7.3	0.7±5.1	-0.7 [-4.8 to 3.4] ( <i>P</i> =0.72)
Adiponectin, pg/mL	0.5±3.4	-0.2±4.0	-0.2 [-2.4 to 2.0] ( <i>P</i> =0.84)
F2-isoprostanes, pg/mL	-36±95	94±200	+91 [3 to 178] ( <i>P</i> =0.042)
Tumor necrosis factor-α, pg/mL	-61±70	16±21	+45 [-11 to 101] ( <i>P</i> =0.11)

CI, 0.03 to 0.24]; *P*=0.017). There was no significant change in abdominal, waist, or hip girths, or in subcutaneous abdominal fat and liver fat measured by CT. However, there was a significant decrease in brachial artery FMD (-1.3% [95% CI,

-2.4 to -0.3]; *P*=0.013), and a significant increase in plasma F2-isoprostanes (+91 pg/mL [95% CI, 3 to 178]; *P*=0.042). There was no change in estimated insulin sensitivity, total cholesterol, low-density lipoprotein cholesterol,



Main findings of changes caused by CLA using baseline values as covariates (ANCOVA).

high-density lipoprotein cholesterol, triglycerides, CRP, leptin, or adiponectin.

There was a significant negative correlation between change in F2-isoprostanes and change in total limb skin folds (ie, loss of limb skin-fold thickness was associated with an increase in F2-isoprostanes) for the entire group (CLA + olive oil) ( $P=0.012$ ) but no significant correlation when each group was analyzed separately. There was no significant correlation between change in endothelial function and change in F2-isoprostanes or change in limb skin-fold thicknesses.

## Discussion

Obesity, and in particular abdominal obesity, is associated with increased cardiovascular risk.<sup>22–24</sup> and intentional weight reduction improves cardiovascular risk.<sup>25,26</sup> Recently there has been a great deal of interest in the effect of the Mediterranean diet on cardiovascular risk. A 2-year study examining the effect of the Mediterranean diet in patients with the metabolic syndrome found a reduction in body weight, an improvement in endothelial function, a decrease in CRP and an improvement in insulin resistance.<sup>27</sup> A study that tried to identify which component of the Mediterranean diet was responsible for improving cardiovascular risk paradoxically found that olive oil impaired endothelial function, although this was inversely correlated with changes in triglycerides.<sup>28</sup> The conclusion of the study was that it was the antioxidant and omega-3-rich foods that conferred cardiovascular benefit. Nevertheless, these studies supported the view that dietary modification or supplementation may have a significant impact on obesity and, in particular, cardiovascular risk.

Experimental evidence in animal models suggests that CLA supplementation, in particular the 10,12 CLA isomer, induces fat mass loss.<sup>29–32</sup> On the basis of this initial evidence, there has been a great deal of interest in its use as an aid to lose fat and weight in humans. Blankson et al reported that 12-week supplementation with >3.4 g/d isomeric CLA significantly reduced body fat mass in overweight volunteers, although there was no change in weight or BMI.<sup>13</sup>

and Riserus et al found that only 4-week supplementation with 4.2 g/d isomeric CLA significantly improved sagittal abdominal diameter.<sup>33</sup> However, Zambell et al found no significant change in weight, BMI, or fat mass after 3-g/d supplementation with isomeric CLA (in women who were not overweight).<sup>18</sup> The observation in two studies of an impairment of insulin sensitivity have raised concerns.<sup>12–13</sup> This study was therefore designed to assess the effect of CLA supplementation on BMI, body fat distribution, and markers of cardiovascular risk, including endothelial function.

Our study found that an isomeric mixture of CLA did not cause significant weight loss (although there was a trend to weight loss of 1.1 kg). This is consistent with the 0.24 to 0.46 kg weight loss reported in previous studies.<sup>13,18</sup> Although CLA reduced limb fat, it had no effect on abdominal fat or liver fat (although there was a nonsignificant trend to an increase in liver fat, measured as a decrease in liver density in Hounsfield units). This finding is in contrast to a previous report that found a decrease in sagittal abdominal diameter after 4 weeks of 4.2 g/d CLA.<sup>33</sup> However, the suggestion that CLA may have a lipodystrophic effect is not new. A study supplementing mice with isomeric CLA found a reduction in fat mass, liver hypertrophy, and an increase in insulin resistance,<sup>34</sup> whereas mice fed the 10,12 CLA isomer had hyperinsulinemia and an increase in liver fat develop.<sup>35</sup> The mechanism for this is not clear, although a rapid decrease in leptin and adiponectin has been observed in mice only 2 days after starting CLA supplementation.<sup>36</sup> A decrease in leptin has also been observed in rats.<sup>37</sup> This hypothesis is supported by the observation that hyperinsulinemia and liver steatosis are partially reversed when hypoleptinemia is normalized by leptin infusion in CLA lipotrophic mice.<sup>34</sup> However, there is conflicting evidence regarding the effects of CLA supplementation on plasma leptin in humans. One study supplementing patients with type 2 diabetes with CLA found a decrease in leptin,<sup>18</sup> but another study supplementing obese men with CLA found no change in leptin.<sup>13</sup> No clinical studies in humans have measured adiponectin after CLA supplementation. In contrast to these findings, a study supplementing Zucker diabetic fatty rats with CLA found that the previous impaired glucose tolerance improved.<sup>39</sup>

Cell culture and animal studies have suggested several other potential mechanisms by which CLA may reduce body fat, including reducing apolipoprotein B secretion in HepG2 cells,<sup>40</sup> increasing carnitine palmitoyltransferase activity and decreasing lipoprotein lipase activity,<sup>30</sup> and increasing tumor necrosis factor and uncoupling protein levels.<sup>34</sup> The role of peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) activity is not clear. PPAR $\gamma$  activity was increased in Zucker diabetic fatty rats and genetically obese mice fed CLA.<sup>39,41,42</sup> although several *in vitro* studies have found that 10,12 CLA downregulates PPAR $\gamma$  activity in mice adipocytes.<sup>43,44</sup> *In vivo* and *in vitro* studies in pigs have also found that CLA induced an increase in PPAR $\gamma$  activity.<sup>45</sup> However, human studies have found an opposite effect on PPAR $\gamma$ . Human adipocytes cultured *in vitro* with 10,12 CLA decreased the expression of PPAR $\gamma$ ,<sup>41,43</sup> and diabetic patients treated with PPAR $\gamma$  agonists (glitazones) experience decreased central fat and increased peripheral fat.<sup>46</sup>

Our observations are important because reducing nonabdominal fat is less likely to reduce cardiovascular risk, and an increase in hepatic fat will increase insulin requirements.<sup>47</sup> Thus any weight loss with this regime is at most modest and the pattern of weight loss is not metabolically favorable.

Furthermore, we found that CLA significantly impaired brachial artery endothelial function, consistent with an adverse impact on cardiovascular risk.<sup>51</sup> The mechanism of this effect is not clear. We and others<sup>16</sup> found an increase in  $F_2$  isoprostanes, a lipid peroxidation product generally considered to be a marker of increased oxidative stress. Another previous study reported an increase in plasma CRP, a marker of inflammation.<sup>17</sup> Taken together, these data suggest that this CLA regime impairs endothelial function and that this may, at least in part, be caused by increased oxidative stress. It is possible that the observed change in FMD in this study has been caused by the change in limb skin-fold thicknesses interfering with the FMD technique (for example, by altering wrist arterial occlusion pressure or changing depth from probe to brachial artery), thus giving a false measurement. However, we feel that this is unlikely because a high wrist arterial occlusion pressure was used and a change in depth to the brachial artery of 1 to 2 mm is well within the capability of the ultrasound probe. Brachial artery FMD measurements vary markedly from laboratory to laboratory, dependant on the exact technique used.<sup>48</sup> In this study, we used the wrist cuff technique, which results in lower values of brachial artery FMD than those obtained using the upper arm or mid-forearm techniques. We did not observe an adverse impact on insulin sensitivity. However, the study may have been under-powered to detect differences using the HOMA technique.<sup>52</sup> We did not identify any change in plasma lipid profiles.

The effect of CLA on other cardiovascular risk factors has been examined. One study reported that CLA decreased platelet aggregability,<sup>53</sup> but another reported no change in platelet aggregability.<sup>54</sup> A small decrease in total, low-density lipoprotein, and high-density lipoprotein cholesterol was found in overweight men taking 1.7 g/d CLA and 3.4 g/d CLA. Although this was not maintained at higher doses.<sup>10</sup> A further study in obese men using 10, 12 CLA and a CLA mixture lowered high-density lipoprotein cholesterol, although no change was observed in total or low-density lipoprotein cholesterol or triglycerides.<sup>13</sup> No change in lipids was found in a study with healthy women supplemented with 3.9 g/d CLA.<sup>55</sup>

Consistent with our observations, 4.2 g/d CLA for 1 month was found to increase urinary isoprostanes in men with abdominal obesity.<sup>16</sup> Isoprostanes are produced from peroxidation of lipids, and it was suggested that the increase in isoprostanes might be simply a result of increased fat lipolysis, rather than indicating increased oxidative stress. However, more recent studies have found that 10, 12 CLA increases insulin resistance<sup>15</sup> and plasma CRP.<sup>17</sup> Taken together with our observation of an impairment of endothelial function, it seems highly likely that the increase in isoprostanes does indeed imply an increase in oxidative stress.

## Conclusions

CLA supplementation for 12 weeks using the regime used in this study had no significant effect on BMI. Even if this represents a type 2 error, the reduction is at most modest, consistent with previous reports in man. Furthermore, the pattern of fat loss is peripheral rather than central. Importantly, the observed impairment of endothelial function and increase in markers of oxidative stress raise concerns about the widespread use of this agent until further studies demonstrate its cardiovascular safety or otherwise.

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## ADVERSE CARDIOVASCULAR AND CENTRAL NERVOUS SYSTEM EVENTS ASSOCIATED WITH DIETARY SUPPLEMENTS CONTAINING EPHEDRA ALKALOIDS

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## ABSTRACT

**Background** Dietary supplements that contain ephedra alkaloids (sometimes called ma huang) are widely promoted and used in the United States as a means of losing weight and increasing energy. In the light of recently reported adverse events related to use of these products, the Food and Drug Administration (FDA) has proposed limits on the dose and duration of use of such supplements. The FDA requested an independent review of reports of adverse events related to the use of supplements that contained ephedra alkaloids to assess causation and to estimate the level of risk the use of these supplements poses to consumers.

**Methods** We reviewed 140 reports of adverse events related to the use of dietary supplements containing ephedra alkaloids that were submitted to the FDA between June 1, 1997, and March 31, 1999. A standardized rating system for assessing causation was applied to each adverse event.

**Results** Thirty-one percent of cases were considered to be definitely or probably related to the use of supplements containing ephedra alkaloids, and 31 percent were deemed to be possibly related. Among the adverse events that were deemed definitely, probably, or possibly related to the use of supplements containing ephedra alkaloids, 47 percent involved cardiovascular symptoms and 18 percent involved the central nervous system. Hypertension was the single most frequent adverse effect (17 reports), followed by palpitations, tachycardia, or both (13); stroke (10); and seizures (7). Ten events resulted in death, and 13 events produced permanent disability, representing 26 percent of the definite, probable, and possible cases.

**Conclusions** The use of dietary supplements that contain ephedra alkaloids may pose a health risk to some persons. These findings indicate the need for a better understanding of individual susceptibility to the adverse effects of such dietary supplements. (N Engl J Med 2000;343:1833-8.)

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**D**IETARY supplements that contain ephedra alkaloids (also known as ma huang) and guarana-derived caffeine are widely consumed in the United States for purposes of weight reduction and energy enhancement. A number of reports of adverse reactions to dietary supplements that contain ephedra alkaloids, some of which resulted in permanent injury or death, have appeared in the medical literature.<sup>1-6</sup> In response to

growing concern about the safety of ephedra alkaloids in dietary supplements, the Food and Drug Administration (FDA) requested an independent review of reports of adverse events related to the use of ephedra alkaloids to assess causation and determine the level of risk these products pose to consumers.

We conducted an in-depth review of 140 reports of adverse events involving dietary supplements containing ephedra alkaloids that were submitted to the FDA between June 1, 1997, and March 31, 1999, and applied a standardized rating system for assessing causation. We also evaluated factors that might increase the risk to consumers and the adequacy of warnings about potential risks included on product labels. The full report of our review of adverse events is available elsewhere.<sup>7</sup> Here, we summarize our findings.

## METHODS

The objective of the review was to determine the likelihood that ephedra alkaloids (which were usually combined with caffeine) caused the reported adverse events on the basis of the information provided in the FDA MedWatch report, along with supplemental medical records. We independently reviewed each of the 140 cases. Causation was assessed according to the criteria described by Blanc et al.<sup>8</sup> and included an evaluation of the timing of the event in relation to the dose and duration of use of a product; an assessment of the pattern of response to determine whether it constituted a recognized reaction to the substance on the basis of previous reports of ephedrine or similar stimulants in the medical literature; and a determination of the contribution of any underlying diseases or medical conditions.

In general, we defined an adverse event as definitely related to the use of supplements containing ephedra alkaloids only if the symptoms recurred with the reintroduction of ephedra alkaloids or when the onset of symptoms coincided with the expected peak plasma concentration of the drug and resolved within an interval that was consistent with the expected duration of the effect of ephedrine. An adverse event was defined as probably related to the use of supplements containing ephedra alkaloids when the majority of the evidence supported the existence of a causal link but one or more aspects of the case, such as time since the last dose, were unknown or there was a minor inconsistency in the supporting evidence, such as a low reported dose. An adverse event was designated as possibly related to the use of supplements containing ephedra alkaloids when it was equally likely that the adverse event was not related to the use of ephedra alkaloids; for example, in the case of effects that have not been reported in the literature in association with ephedra alkaloids but that are pharmacologically plausible. Reports of ad-

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verse events that included scant medical history and incomplete information about the product involved were usually considered to have insufficient information to be assessed. This category was reserved for events in which the evidence was not substantial enough to consider them as possibly related to the use of supplements containing ephedra alkaloids. Adverse events were defined as probably unrelated to the use of supplements containing ephedra alkaloids if the evidence that ephedra alkaloids were the cause was weak or if the likelihood was strong that there was some other cause, either medical or toxicologic. When the scientific evidence or course of events was highly inconsistent with the known effects of ephedra alkaloids, the event was considered definitely unrelated; for example, in the case of symptoms that persisted long after the use of ephedra alkaloids had been discontinued or in the case of symptoms that had no association with the known pharmacodynamic effects of ephedra alkaloids. However, the event was considered related if the patient had a preexisting condition such as hypertension that could have been aggravated by the use of ephedra alkaloids and if the pattern of use met the criteria for causation.

In determining the likelihood of a causal link, we evaluated aspects of the medical history, dietary patterns, and social habits as possible contributing or causative factors. For example, we noted when events occurred while patients were fasting or in conjunction with high intakes of caffeine. We recognized that in the case of adverse events that were most likely not related to the use of supplements containing ephedra alkaloids one or more of the other ingredients in the supplement may have been causally related to the event.

## RESULTS

### Features of the Cases

The age and sex of the users of products containing ephedra alkaloids and the reported reasons for use are shown in Table 1. Although the labels of most such products state that they are not intended for use by persons less than 18 years of age, adverse events were recorded in at least 10 persons under this age. The youngest was 15 years old. Overall, 43 cases (31 percent) were considered to be definitely or probably related to the use of supplements containing ephedra alkaloids, 24 cases (17 percent) were considered to be unrelated to the use of such supplements, 44 cases (31 percent) were deemed possibly related to the use of such supplements, and in 29 cases (21 percent) the information provided was insufficient to assess causation. The types of adverse events that were definitely or probably related to the use of supplements containing ephedra alkaloids and those that were possibly related are summarized in Table 2.

Cardiovascular symptoms made up 47 percent of the adverse events that were definitely, probably, or possibly related to the use of supplements containing ephedra alkaloids. Hypertension was the single most frequent adverse effect, followed by palpitations, tachycardia, or both. Eighteen percent of related and possibly related adverse events involved the central nervous system. Strokes ( $n=10$ ) and seizures ( $n=7$ ) were the most frequent type of central nervous system event reported. The clinical outcomes of the definite, probable, and possible cases are listed in Table 3.

Ten events resulted in death (including 1 neonatal death and 1 fetal death), and 13 events resulted in permanent impairment, which represented 26 percent of

**TABLE 1. AGE AND SEX OF USERS OF SUPPLEMENTS CONTAINING EPHEDRA ALKALOIDS AND REASONS FOR USE, ACCORDING TO ADVERSE-EVENT REPORTS.**

VARIABLE	No. of Users (%)
Age	
<18 yr	10 (7)
18–29 yr	34 (24)
30–45 yr	45 (32)
>45 yr	32 (23)
Unknown	19 (14)
Sex	
Male	56 (40)
Female	84 (60)
Reason for use	
Weight loss	83 (59)
To increase athletic performance	23 (16)
To increase energy	9 (6)
Unknown	24 (17)
Intentional misuse	1 (1)

the definite, probable, and possible cases. Nine serious adverse events occurred in persons who were taking relatively low doses of ephedra alkaloids (range, 12 to 36 mg per day) and who had no important medical risk factors. Features of the definite or probable and possible cases that resulted in death or permanent impairment or that necessitated substantial medical intervention are given in Tables 4 and 5, respectively.

Of the sudden catastrophic cerebrovascular and cardiovascular events, 11 occurred in previously healthy persons. Some of these cases, which were definitely or probably related to the use of supplements containing ephedra alkaloids, are described in detail in the following sections.

### Examples of Severe Cerebrovascular Adverse Events

#### Patient 1

Patient 1 was a healthy 35-year-old woman who had taken aerobic-exercise classes for several years without incident (Table 4). In July 1997, she began taking one capsule of Shape-Fast Plus (according to the label, each capsule contained 15 mg of ephedra alkaloids and 40 mg of caffeine) three times a day before meals for weight loss; she was taking no other medications. She had been taking the product for one week when she collapsed during an aerobics class. Bystanders observed that her arms and legs were flexing and tensing. In the emergency department, her blood pressure was 110/38 mm Hg and the heart rate was 104 beats per minute. A computed tomographic scan of the head showed a subarachnoid hemorrhage. Cerebral angiography showed no evidence of a vascular aneurysm. A urine toxicology screen was positive for amphetamine, a result presumed to reflect a cross-reaction with the ephedrine and therefore to be false positive.

**TABLE 2. TYPES OF ADVERSE EVENTS THAT WERE DEFINITELY, PROBABLY, OR POSSIBLY RELATED TO THE USE OF SUPPLEMENTS CONTAINING EPHEDRA ALKALOIDS.**

ADVERSE EVENT	DEFINITELY OR PROBABLY RELATED (N=43)	POSSIBLY RELATED (N=44)	TOTAL (N=87)
	no. of events (%)		
Cardiovascular			
Hypertension	10 (21)	7 (14)	17 (17)
Palpitations, tachycardia, or both	8 (17)	5 (10)	13 (13)
Arrhythmias	3 (6)	3 (6)	6 (6)
Myocardial infarction	2 (4)	0	2 (2)
Cardiac arrest or sudden death	5 (10)	3 (6)	8 (8)
Central nervous system			
Stroke	4 (8)	6 (12)	10 (10)
Transient ischemic attack	1 (2)	0	1 (1)
Seizure	1 (2)	6 (12)	7 (7)
Other	14 (29)	20 (40)	34 (35)
Total no. of events*	48	50	98

\*The total number of events exceeds the total number of cases, since some cases involved more than one adverse event.

Neurogenic pulmonary edema rapidly developed, necessitating endotracheal intubation and mechanical ventilation. Electrocardiographic findings and cardiac-enzyme levels were consistent with the occurrence of a small myocardial infarction. The treating cardiologist and neurologist thought that ephedrine induced the subarachnoid hemorrhage. The finding of amphetamine on the urine toxicology test supports the presence of ephedrine at the time of the event. Laboratory analysis of the supplement determined that the ephedrine content was 12.0 mg per capsule. At that time, the FDA's recommendation was a maximal dose of 8 mg per serving.<sup>7</sup>

#### Patient 10

Patient 10 was an apparently healthy 39-year-old man who experienced numbness of the right arm and leg on March 17, 1998, 90 minutes after drinking Ultimate Orange, which according to the label contained 415 mg of ma huang (ephedra alkaloids) per serving as well as guarana (a source of caffeine), and 5 minutes after running 3 miles (4.8 km) (Table 4). He also regularly took multivitamins and amino acid supplements, but no other medications. On presentation at a nearby hospital, his blood pressure was 140/78 mm Hg and his pulse was 60 beats per minute. A computed tomographic scan of the head revealed a left-sided intrathalamic hemorrhage. Cerebral angiography showed no evidence of vascular anomalies. The patient had gradual clinical improvement, and his symptoms resolved except for persistent sensory loss on the right side of his face. Chemical analy-

**TABLE 3. CLINICAL OUTCOMES OF ADVERSE EVENTS THAT WERE DEFINITELY, PROBABLY, OR POSSIBLY RELATED TO THE USE OF SUPPLEMENTS CONTAINING EPHEDRA ALKALOIDS.**

OUTCOME	DEFINITELY OR PROBABLY RELATED (N=43)	POSSIBLY RELATED (N=44)	TOTAL (N=87)
	no. of events (%)		
Death	3 (7)	7 (16)	10 (11)
Permanent impairment	7 (16)	6 (14)	13 (15)
Ongoing medical treatment	4 (9)	4 (9)	8 (9)
Full recovery	29 (67)	13 (30)	42 (48)
Unknown	0	14 (32)	14 (16)

sis of the Ultimate Orange product confirmed the presence of ephedrine, as well as of pseudoephedrine, norephedrine, and norpseudoephedrine.

#### Examples of Severe Cardiovascular Adverse Events

##### Patient 2

Patient 2 was a 22-year-old man with a history of asthma who collapsed while lifting weights at a gym on March 31, 1998 (Table 4). His medications included theophylline (Theo-Dur; 300 mg twice daily), albuterol (Ventolin; administered as necessary through a metered-dose inhaler), and a combination of chlorpheniramine maleate, phenylephrine hydrochloride, and phenylpropanolamine hydrochloride (Atrohist Plus SR). According to friends, he had consumed one 18-oz bottle of Ripped Force (which is listed as containing 20 mg of ephedrine alkaloids, 100 mg of caffeine, 250 mg of L-carnitine, and 240 µg of chromium) before working out and was regularly drinking three bottles of Ripped Force per day. He also took creatine and protein supplements. Witnesses reported that he had a seizure. Paramedics initially found him apneic and in ventricular fibrillation. He was successfully resuscitated. Computed tomography of the head showed cerebral edema but no hemorrhage or masses. An initial electrocardiogram showed atrial flutter, which subsequently converted to sinus rhythm. An echocardiogram revealed mild left ventricular hypertrophy. The plasma theophylline level was 11 µg per milliliter (therapeutic range, 10 to 20), and urinalysis revealed 12 µg of ephedrine per milliliter, 0.38 µg of pseudoephedrine per milliliter, and 0.41 µg of phenylpropanolamine per milliliter. The treating cardiologist thought that the combination of ephedra alkaloids and caffeine in Ripped Force and the theophylline and albuterol medications caused a ventric-

TABLE 4. OUTCOME IN 11 PATIENTS WITH ADVERSE EVENTS THAT WERE DEFINITELY OR PROBABLY RELATED TO THE USE OF SUPPLEMENTS CONTAINING EPHEDRA ALKALOIDS.

PATIENT NO.	AGE (YR)/SEX	NAME OF SUPPLEMENT	ESTIMATED DAILY DOSE OF EPHEDRA ALKALOIDS mg	DURATION OF USE	ADVERSE EVENT	OUTCOME	PREEXISTING CONDITIONS OR CONCURRENT RISKS
1	35/F	Shape-East Plus	45	1 wk	Subarachnoid hemorrhage	Permanent disability	None
2	22/M	Ripped Force	20–60	Unknown	Arrhythmia, cardiac arrest	Permanent disability	Asthma
3	28/F	Herbalife's Thermojetix	21	1 day	Cardiac arrest	Permanent disability	None
4	43/M	Ripped Fuel	60	7 mo	Cardiac arrest	Death	Family history of coronary artery disease
5	37/F	Metabolife 356	36	1 wk	Severe hypertension, cardiac arrest, hypokalemia	Death	None
6	59/F	OmniTrim Extra Vitamin-Formulated	36	3 wk	Acute myocardial infarction	Coronary bypass surgery	Hypertension
7	38/M	Ripped Fuel	20	1 yr	Arrhythmia, cardiac arrest	Death	None
8	47/F	Total Control	44–66	9 mo	Hypertension, bilateral lacunar infarctions	Permanent disability	Concomitant ingestion of caffeine and ethanol
9	29/M	Ultimate Orange	30	2 wk	Stroke	Permanent disability	Concomitant use of dehydroepiandrosterone and androstenedione
10	39/M	Ultimate Orange	Unknown	Unknown	Hemorrhagic stroke	Permanent disability	None
11	47/M	Purple Blast	Unknown	3 wk	Hemorrhagic stroke	Permanent disability	Possible hypertension

ular arrhythmia that resulted in cardiac arrest. The patient suffered anoxic encephalopathy and remained in a vegetative state for several weeks. After one month in an acute care facility and six weeks at a rehabilitation facility, he was discharged with substantial residual neurologic impairment.

#### Patient 7

Patient 7 was an apparently healthy 38-year-old man who had been taking two capsules of Ripped Fuel (according to the label each capsule contains 10 mg of ephedrine and 100 mg of caffeine) each morning for one year as directed on the product label (Table 4). On June 6, 1996, he took his usual dose along with a cup of coffee and went jogging for 20 minutes. After returning home, he was talking with his family when he suddenly collapsed and appeared to have a tonic-clonic seizure. He had not reported any symptoms before collapsing. He was in full cardiac arrest when paramedics arrived and could not be resuscitated. Autopsy showed mild cardiomegaly with four-chamber dilatation and coronary artery disease, with narrowing of 50 to 75 percent in four vessels. The cause of death was acute arrhythmia resulting from atherosclerotic cardiovascular disease. Subsequent toxicology testing showed blood levels of 110 ng of ephedrine per milliliter (the therapeutic range used for bronchodilation is 20 to 80).<sup>9</sup> An addendum

to the autopsy report included the comment, "ephedrine is a stimulant medication, and as such may have contributed to a fatal arrhythmia in the decedent."<sup>9</sup>

#### DISCUSSION

Ephedrine and related alkaloids have been associated with adverse cardiovascular events, including acute myocardial infarction, severe hypertension, myocarditis, and lethal cardiac arrhythmias.<sup>10,11</sup> Constriction of coronary arteries and, in some cases, vasospasm are believed to be the mechanisms of myocarditis and myocardial infarction. The adrenergic effects of ephedrine shorten cardiac refractory periods, permitting the development of reentrant cardiac arrhythmias. Ephedrine can predispose patients to both hemorrhagic and ischemic stroke.<sup>12</sup> Subarachnoid hemorrhage is thought to be a result of the hypertensive action of ephedrine, which can be short lived, or of cerebral vasculitis, which has been described in association with a variety of sympathomimetic drugs.<sup>13,14</sup> Thrombotic stroke is presumably related to vasoconstriction of large cerebral arteries, which leads to local thrombosis as a result of stasis and sympathomimetic-induced platelet activation.

Caffeine is present in many products that contain ephedra alkaloids, and those who take these products might also be consuming considerable quantities of caffeine in coffee, tea, and soft drinks. Caffeine is like-

TABLE 5. OUTCOME IN 15 PATIENTS WITH ADVERSE EVENTS THAT WERE POSSIBLY RELATED TO THE USE OF SUPPLEMENTS CONTAINING EPHEDRA ALKALOIDS.

PATIENT No.	AGE (yr)/SEX	NAME OF SUPPLEMENT	ESTIMATED DAILY DOSE OF EPHEDRA ALKALOIDS mg	DURATION of Use	ADVERSE EVENT	OUTCOME	PREEXISTING CONDITIONS OR CONCURRENT RISKS
1	46/M	Diet Fuel	Unknown	5-6 mo	Stroke	Death	None
2	22/M	Ripped Fuel	Unknown	Unknown	Hypertension, abnormal electrolyte levels, cardiac arrest	Death	None
3	64/F	Fit America Natural Weight Control Aid	Unknown	2 mo	Atrial fibrillation, stroke	Permanent disability	Hypertension, transient ischemic attack
4	47/F	Per-Form Dieter's Natural Tea	Unknown	6 mo	Rhabdomyolysis, hyponatremia, hypokalemia	Prolonged hospital care	None
5	64/F	Shape-Fast	20	Unknown	Hemorrhagic stroke	Permanent disability	None
6	34/M	Herbalife's Thermojetics	Unknown	>3 wk	Atrial flutter, renal failure, hypokalemia, rhabdomyolysis	Death	None
7	32/F	Ripped Fuel	20	4 yr	Premature delivery (34 wk of gestation)	Death of neonate	Smoking
8	29/M	Ultimate Nutrition Product Ma Huang	Unknown	6-7 mo	Stroke	Permanent disability	None
9	15/F	Ripped Fuel	Unknown	2-3 wk	Arrhythmia, cardiac arrest	Death	None
10	41/F	Diet-Then	12	1 mo	Hypertension, multiple brainstem infarcts	Permanent disability	Possible hypertension
11	22/F	Magic Herb	72	3 mo	Spontaneous abortion at 9 wk	Death of fetus	None
12	43/F	Metabolic 356	12	6 mo	Severe hypertension, hemorrhagic stroke	Permanent disability	None
13	18/M	Ultimate Orange	Unknown	Unknown	Seizure, hemorrhagic stroke	Death	None
14	61/F	Metabolic 356	24; increased to 60	1 mo	Hypertension, unstable angina	Coronary bypass surgery	Asthma
15	26/M	Ripped Fuel	20-60	3 yr	Status epilepticus, hypokalemia	Permanent disability	None

ly to enhance the cardiovascular and central nervous system effects of ephedrine. Caffeine acts by competitively antagonizing the receptors for adenosine, a hormone released by endothelial cells that dilates blood vessels.<sup>15</sup> By inhibiting adenosine-mediated dilatation of blood vessels, caffeine constricts blood vessels and may increase blood pressure in persons prone to hypertension. Caffeine also augments the release of catecholamines, an effect that, when combined with that of ephedrine, could lead to increased stimulation of the central nervous system and cardiovascular system.<sup>16</sup>

Phenylpropanolamine, another ephedrine alkaloid, was marketed with caffeine in various weight-reducing aids until 1983, when the combination was banned by the FDA after numerous reports of adverse effects. Several studies have shown that caffeine and phenylpropanolamine have an additive effect on blood pressure.<sup>17</sup> These interactions between phenylpropanolamine and caffeine support the idea that the combination of ephedrine and caffeine in a dietary supplement could increase the risk of adverse effects.

The quantity of ephedrine in dietary supplements,

as reported on package labels, is typically about 20 mg per serving, and the usual dose frequency is two to three times per day. These products may contain larger or smaller amounts of ephedra alkaloids than are listed on the product label. For example, 11 of 20 supplements tested by Gurley et al.<sup>18</sup> either failed to list the alkaloid content on the label or had more than a 20 percent difference between the amount listed on the label and the actual amount.

Often, the dose of ephedrine that was associated with an adverse event was less than a typical dose of ephedrine used for bronchodilation (25 to 50 mg). Experimental studies show that ephedrine has only moderate effects on heart rate and blood pressure at these doses.<sup>19,20</sup> The discrepancy between such data and our findings of serious adverse events reported with the use of dietary supplements containing ephedra alkaloids may be due to individual susceptibility, the additive stimulant effects of caffeine, the variability in the contents of pharmacologically active chemicals in the products, or preexisting medical conditions.

Many of the cases we reviewed involved side effects

such as anxiety, tremulousness, insomnia, palpitations, and personality changes that are well known to occur with the use of stimulant drugs. When ephedrine is used for medical purposes, these types of reactions are considered side effects and must be included in the assessment of risks and benefits. In fact, ephedrine is rarely prescribed today for medical purposes, because newer drugs have more specific actions and fewer side effects. The risks of taking ephedra alkaloids as a dietary supplement, however, are difficult to justify because the alkaloids have no demonstrated benefit. Unlike vitamins and minerals, ephedra alkaloid supplements are not essential for proper nutrition. People who take these products to increase their exercise capacity or to lose weight place themselves at risk without a substantial likelihood of benefit.

A limitation to the use of reports of adverse events as an indicator of a product's safety is that the number of people at risk for the event is unknown. Manufacturers of dietary supplements that contain ephedra alkaloids reported that 3 billion servings were sold in 1999.<sup>24</sup> The number of servings that were actually consumed is difficult to determine. Assuming that the products were consumed as directed — three doses per day for 12 weeks — then approximately 12 million people used these supplements in 1999.

Another limitation is that adverse events are known to be underreported. Several studies have shown that spontaneous reporting of adverse events to MedWatch is not routine, and the rate of reporting may be less than 15 percent.<sup>24,25</sup> The frequency of reports of adverse reactions to herbal products is thought to be even lower.<sup>24</sup> Therefore, the frequency of serious adverse events associated with the use of supplements containing ephedra alkaloids cannot be precisely determined with the use of current reporting mechanisms.

Because of the severity of the adverse events that we reviewed and, in particular, the occurrence of events that caused permanent disability and death, we conclude that dietary supplements that contain ephedra alkaloids pose a serious health risk to some users. Although the incidence of serious adverse effects cannot be determined from our analysis, our findings arouse concern about the risks of these products, given that they have no scientifically established benefits. Our findings indicate the need for a better understanding of the determinants of individual susceptibility to the serious adverse effects of dietary supplements containing ephedra alkaloids so that appropriate dosing guidelines and warnings can be devised.

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**X. RELATED PROCEEDINGS APPENDIX**

No decisions have been rendered by a court or the Board in any proceeding identified pursuant to paragraph (c)(1)(ii) C.F.R. §41.37.

**XI. CONCLUSION**

For the foregoing reasons, Appellants respectfully submit that the Examiner's rejection of Claims 1-3, 7 and 9 is erroneous. Reversal of the rejections is respectfully requested. Appellants request that the Board render a decision as to the allowability of the Claims.

Respectfully submitted,

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